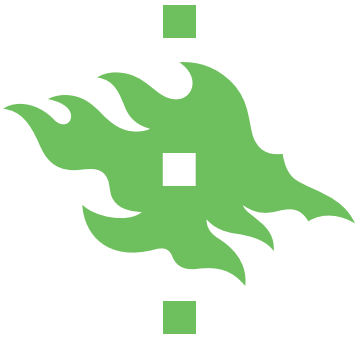

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

ROBERT LEIGH

CHARACTERIZATION AND SELECTIVE MODULATION OF CHAMBER-SPECIFIC GENE REGULATORY NETWORKS UNDERLYING CONGENITAL AND ADULT HEART DISEASE

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Characterization and selective modulation of chamber-specific gene regulatory networks underlying congenital and adult heart disease

Robert Leigh

ACADEMIC DISSERTATION

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Abstract

Disruption of chamber-specific gene regulatory networks underlies malformation of the heart and results in congenital heart disease. Re-activation of fetal gene regulatory networks in adults occurs during cardiac injury, and transcription factors composing these networks represent candidate therapeutic targets to impede heart failure progression. The identification of molecular markers and underlying regulators of cardiac chamber specification is thus an important step in understanding the aetiology of cardiovascular disease. Moreover, the examination of chemical modulation of molecular processes occurring during development allows for a more detailed understanding of the teratogenic effects of chemical compounds and could lead to the development of small molecule-based strategies for stimulating or impeding well-characterized developmental processes in the adult heart. To this end, this thesis is comprised of three studies related to the differentiation of atrial and ventricular cardiomyocytes and the selective modulation of this process.

Study I led to the generation of an *in vitro* model of cardiomyocyte subtype specification of pluripotent stem cells for the use in studies II-III. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of native embryonic atrial and ventricular tissue confirmed the robust ventricular-specific expression of myosin light chain 2 (Myl2) and also indicated the lack of an endogenous atrial-specific marker during early embryogenesis. Genome editing was used to integrate a fluorescent reporter into the endogenous Myl2 locus to mark cells of the ventricular lineage, whereas atrial cells were traced by an atrial-specific transgene driven by the slow myosin heavy chain 3 (SMyHC3) promoter. Atrial and ventricular reporter expression were confirmed *in vivo* by laser-assisted morula injection of reporter mouse embryonic stem cells (mESCs) and microscopy of chimeric embryos.

In addition to this *in vivo* validation, spontaneous differentiation of reporter mESCs was characterized by qRT-PCR, indicating dynamic expression of retinoic acid signalling components. Differentiation assays were developed based on chemical perturbation of undifferentiated progenitor cells and differentiated cardiomyocytes, respectively. Members of the retinoid family, known teratogens and modulators of anterior-posterior patterning, were tested for effects on the activation of atrial and ventricular reporter genes. Additionally, a directed-differentiation assay was developed based on highly pure multipotent progenitor cells and differentiation assessment in a 384-well format. In this assay, chemical inhibitors of Wnt and Transforming growth factor β (Tgfβ) pathways led to promotion of ventricular reporter expression when added at the multipotent progenitor stage, but not after the onset of spontaneous beating. Additionally, exogenous *all-trans* retinoic acid added to undifferentiated progenitors led to an inhibition of ventricular differentiation, whereas addition following the onset of spontaneous beating led to activation of the ventricular reporter gene.

In addition to chemical probes described in study I, novel compounds targeting the protein-protein interaction of core cardiac transcription factors Gata transcription factor 4 (Gata4) and NK2 homeobox 5 (Nkx2-5) were examined in study II. Specifically, the effects of GATA-targeted compounds on the differentiation of atrial and ventricular cardiomyoyctes were explored. Lead compound 3i-1000 increased the proportion of atrial and ventricular reporter cells after 10-day treatment in the spontaneous differentiation assay. Further exploration of the effects of GATA4 targeted compounds revealed that a shorter treatment (2-day) of cells prior to the onset of spontaneous beating led to an upregulation of ventricular reporter genes in a directed differentiation assay. An acetyl-lysine like domain among active compounds, in addition to analysis of the GATA4 interactome by Bio-ID revealed the potential association of bromodomain-containing proteins with chamber-

specific gene expression. This was further investigated by combinatorial treatment with the Bromo- and Extra- Terminal domain family (BET) bromodomain inhibitor JQ1 and GATA-targeted compounds in reporter gene assays. Finally, the effects of GATA compounds on cardiomyocyte maturation were explored by compound treatment and global run-on sequencing (GRO-seq) in primary cardiomyocytes, revealing the upregulation of several targets previously identified as regulators of cell fate determination and regeneration.

Study III detailed the embryonic/cardiac expression of proCholecystokinin (proCCK), a classical gut and neuropeptide. Analysis of mRNA-seq data suggested that proCCK is a transcriptional target of the TBX5 transcription factor in mESC-derived cardiomyocytes. Native, endogenous mRNA levels were characterized in embryonic hearts by whole mount *in situ* hybridization and optical projection tomography, revealing that proCck mRNA is present prior to the linear heart tube stage and that it is upregulated in the ventricles compared to the atria in the newly formed embryonic heart. Interestingly, mRNA of proCck and its receptors Cckar/Cckbr is mostly restricted to the atrial chambers in neonatal stages, in line with its potential role in regulating cardiac rhythm. *In silico* analysis implicated both TBX5 and MEF2C as regulators of proCck transcription, and this regulation was confirmed by conducting *in vitro* reporter gene assays. Additionally, proCCK was induced by endothelin-1 (ET-1), another peptide associated with maladaptive remodelling during heart failure. Furthermore, proCck mRNA levels declined in the left ventricles of rats following myocardial infarction (MI). Finally, exogenous cholecystokinin octapeptide (CCK-8) exerted no effects on the differentiation process of pluripotent stem cells (PSCs) to the cardiomyocyte fate.

Collectively, these studies led to the generation of new methodology for the study of chamber-specific cardiac gene regulatory networks. Additionally, they led to an improved understanding of the dynamics of chamber-specific marker localization and upstream transcription factors governing their expression, potentially important to biomarker development. Finally, these studies have indicated that specific chemical compounds are capable of influencing chamber-specific gene regulatory networks. This knowledge might be utilized to develop novel therapeutic strategies for the treatment of heart failure.

Tiivistelmä

Geenisäätelyverkostojen häiriöt kammioissa ja eteisissä aiheuttavat niille tyypillisiä sydämen epämuodostumia ja synnynnäisiä sydänsairauksia. Aikuisten sydänlihasvaurioissa havaitaan sikiöaikaisten geenisäätelyverkostojen uudelleenaktivoitumista, joten näihin verkostoihin liittyvät transkriptiotekijät ovat mahdollisia lääkevaikutuksen kohteita sydämen vajaatoiminnan etenemisen hidastamisessa. Sydäntautien etiologian ymmärtämisen kannalta onkin oleellista tunnistaa spesifisesti kammioiden ja eteisten erilaistumista sääteleviä molekulaarisia tekijöitä. Lisäksi kemiallisten yhdisteiden molekulaaristen vaikutusten tutkiminen sydämen kehityksen aikana lisää tietoa niiden teratogeenisista ominaisuuksista, mikä voi johtaa uusien pienimolekyylisten yhdisteiden kehittämiseen kehitysbiologisten mekanismien aktivoimiseksi tai estämiseksi aikuisten sydämessä. Tämä väitöskirja koostuu kolmesta tutkimuksesta, jotka keskittyvät sydämen eteisten ja kammioiden sydänlihassolujen erilaistamiseen ja niiden erilaistumisen valikoivaan säätelyyn.

Tutkimuksessa I kehitettiin *in vitro*-menetelmä pluripotenttien kantasolujen erilaistamiseksi eteis- ja kammiolihassoluiksi tutkimuksiin II-III. Alkion eteis- ja kammiokudoksen kvantitatiivinen reaaliaikainen polymeraasiketjureaktio (qRT-PCR)-analyysi vahvisti myosiinin kevyen ketjun 2 (Myl2) voimakkaan tarkkarajaisen ilmentymisen kammioissa ja osoitti myös endogeenisen eteisspesifisen merkkigeenin puuttumisen varhaisen alkion kehityksen aikana. Kammiosolujen tunnistamiseksi käytettiin geenimuokkausta integroimalla fluoresoiva merkkigeeni endogeeniseen Myl2-lokukseen, eteissolut puolestaan jäljitettiin eteisspesifisellä merkkigeenillä, jota ohjasi hidas myosiinin raskasketju 3 (SMyHC3)-promoottori. Eteisen ja kammion merkkigeenien ilmentyminen vahvistettiin *in vivo* laseravusteisella injektiolla morula-vaiheessa reportterihiiren alkion kantasoluihin (mESC) ja kimeeristen alkioiden mikroskopialla.

Tämän *in vivo* -validoinnin lisäksi hiiren alkion kantasolujen, joihin oli integroitu merkkigeenit, spontaania erilaistumista tutkittiin qRT-PCR:llä, jolloin havaittiin ilmentymismuutoksia retinoiinihapon signaalitransduktiojärjestelmässä. Erilaistumismenetelmät kehitettiin perustuen kemiallisten yhdisteiden vaikutuksiin erilaistumattomiin esisoluihin ja erilaistuneisiin sydänlihassoluihin. Retinoidien, jotka ovat tunnettuja teratogeenejä ja säätelevät sydämen anteriorista -posteriorista akselia, vaikutuksia tutkittiin eteis- ja kammiomerkkigeenien aktiivisuuteen. Lisäksi kehitettiin kohdennettu erilaistamismenetelmä, joka perustui pelkkiin monipotentteisiin esisoluihin ja erilaistumisen arviointiin 384-kuoppalevyillä. Tässä menetelmässä Wnt:n ja transformoivan kasvutekijän β (Tgfβ) signaalireittien estäjät lisäsivät kammiomerkkigeenin ilmentymistä, kun ne lisättiin monipotenttisiin esisoluihin, mutta ei spontaanin sykkimisen alkamisen jälkeen. Lisäksi erilaistumattomiin esisoluihin lisätty eksogeeninen *all-trans*-retinoiinihappo esti kammioerilaistumista, kun taas spontaanin sykkimisen alkamisen jälkeen tapahtunut lisäys aktivoi kammiomerkkigeenin.

Tutkimuksessa I kuvattujen pienimolekyylisten yhdisteiden lisäksi tutkimuksessa II tutkittiin uusia yhdisteitä, jotka vaikuttavat sydämen keskeisten transkriptiotekijöiden Gata4 ja Nkx2-5-proteiini-proteiini-vuorovaikutukseen. Erityisesti tutkittiin GATA4-kohdennettujen yhdisteiden vaikutuksia erikseen eteisten ja kammioiden sydänlihassolujen erilaistumiseen. Johtoyhdiste 3i-1000 lisäsi eteisen ja kammion merkkigeenisolujen osuutta 10 vuorokauden hoidon jälkeen spontaanissa erilaistamismenetelmässä. GATA4-kohdennettujen yhdisteiden vaikutusten jatkotutkimus osoitti, että lyhyempi solujen käsittely (2 vuorokautta) ennen spontaanin sykkimisen alkamista aktivoi kammioreportterigeenin kohdennetussa erilaistamismenetelmässä. Aktiivisten yhdisteiden asetyylilysiinin kaltainen domeeni, yhdessä GATA4-vuorovaikutus Bio-ID-analyysin kanssa, tuki

bromodomeeniproteiinien mahdollista yhteyttä kammiospesifiseen geenien ilmentymiseen. Tätä yhteyttä tutkittiin edelleen merkkigeenimäärityksissä BET (Bromo- ja Extra-Terminal domain) bromodomeeniestäjän JQ1 ja GATA4-kohdennettujen yhdisteiden yhdistelmähoidolla. Lopuksi tutkittaessa GATA-kohdennettujen yhdisteiden vaikutuksia sydänlihassolujen kypsymiseen primaarisissa sydänlihassoluissa GRO-sekvensoinnilla (global run-on sequencing), havaittiin useiden solujen kehitykseen ja uusiutumiseen liittyvien geenien ilmentymisen lisääntyvän.

Tutkimuksessa III kuvattiin prokolekystokiniinin (proCCK), klassisen suolisto- ja neuropeptidin, ilmentyminen alkiossa/sydämessä. mRNA-sekvenssitietojen analysointi viittasi siihen, että proCCK on hiiren alkion kantasoluista erilaistetuissa sydänlihassoluissa TBX5-transkriptiotekijän kohde. Kun endogeenisiä mRNA-tasoja tutkittiin alkion sydämissä *in situ* -hybridisaatiolla ja optisella projektiotomografialla, huomattiin että proCck-mRNA ilmentyy ennen lineaarista sydänputkivaihetta ja että sen ilmentyminen paikantui kehittyvässä hiiren alkion sydämessä lähinnä kammioihin verrattuna eteisiin. Kiinnostavaa oli myös, että sekä proCck:n että sen reseptorien, Cckar/Cckbr, mRNA rajoittui vastasyntyneisyysvaiheessa pääasiassa eteisiin, yhdenmukaisesti aikaisempien havaintojen kanssa joissa on todettu proCck:n säätelevän sydämen rytmiä. *In silico* -analyysi viittasi sekä TBX5:n että MEF2C:n merkitykseen proCck-transkription säätelijöinä; tämä löydös vahvistettiin *in vitro* merkkigeenimäärityksien avulla. Endoteliini-1 (ET-1), joka myös on yhdistetty sydämen vajaatoiminnan aikana tapahtuviin sydänlihaksen rakennemuutoksiin, indusoi proCCK:ta. Lisäksi havaittiin, että rottien vasemmassa kammiossa proCck-mRNA-tasot pienenivät kokeellisen sydäninfarktin jälkeen. Lopuksi todettiin, että kolekystokiniinioktapeptidi (CCK-8) ei vaikuttanut pluripotenttien kantasolujen erilaistumiseen sydänlihassoluiksi.

Kokonaisuudessaan tutkimukset johtivat uuden menetelmän kehittämiseen sydämen kammiospesifisten geenisäätelyverkostojen tutkimiseen. Lisäksi tutkimustulokset lisäsivät käsitystä kammiospesifisten tekijöiden ajallisista ja paikallisista muutoksista sekä niiden ilmentymistä säätelevistä ylävirran transkriptiotekijöistä, mikä voi olla tärkeää biomarkkerien kehittämiselle. Lopuksi nämä tutkimukset osoittivat, että tietyt kemialliset yhdisteet vaikuttavat valikoivasti kammiospesifisiin geenisäätelyverkostoihin. On mahdollista, että tätä tietoa voitaisiin hyödyntää kehittäessä uusia lääkehoitoja sydämen vajaatoiminnan hoitoon.

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Original publications

This thesis is based on the following original publications:

I. **Leigh RS**, Ruskoaho HJ, Kaynak BL (2020) A novel dual reporter embryonic stem cell line for toxicological assessment of teratogen-induced perturbation of anterior-posterior patterning of the heart. Arch Toxicol 94: 631-645 doi: 10.1007/s00204-019-02632-1

II. Välimäki MJ*, **Leigh RS***, Kinnunen SM, March AR, Hernandez de Sande A, Kinnunen M, Varjosalo M, Heinäniemi M, Kaynak BL, Ruskoaho H (2021) GATA-targeted compounds modulate cardiac subtype cell differentiation in dual reporter stem cell line. Stem Cell Res Ther 12(1): 190 doi: 10.1186/s13287-021-02259-z

III. **Leigh RS**, Ruskoaho HJ, Kaynak BL (2021) Cholecystokinin peptide signaling is regulated by a TBX5-MEF2 axis in the heart. Peptides 136: 170459 doi: 10.1016/j.peptides.2020.170459

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The publications are referred to in the text by the above roman numerals. Reprints were made with the permission of the copyright holders.

Abbreviations

AKT1	Protein kinase B
ALK2	Activin A Receptor Type 1
ALK3	Bone Morphogenetic Protein Receptor Type 1A
ALK4	Activin A Receptor Type 1B
ALK5	Transforming Growth Factor Beta Receptor 1
ALK6	Bone Morphogenetic Protein Receptor Type 1B
ALK7	Activin A Receptor Type 1C
AMHC1	Atrial-specific myosin heavy chain
ANP	Atrial natriuretic peptide
AP-1	Activator protein 1
ATP2A2	ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2
atrRFP	Atrial-specific (posterior) fluorescent reporter
AV	Atrioventricular
AVC	Atrioventricular canal
BAF	SWI/SNF ATP dependent chromatin remodelling complex
BAF60C	Mammalian Chromatin-Remodeling Complex BRG1-Associated Factor 60C
BET	Bromo- and Extra- Terminal domain family
BioID	proximity-dependent biotin identification
BMP	Bone morphogenetic protein
BMPR1A	Bone morphogenetic protein receptor type 1a
BNP	Brain natriuretic peptide
BRD4	Bromodomain Containing 4
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CCK-8	Cholecystokinin octapeptide
CCKAR	Cholecystokinin A receptor
CCKBR	Cholecystokinin B receptor
CCS	Cardiac conduction system
CDC42	Cell division control protein 42

CDK4	Cyclin-dependent kinase 4
CHD	Congenital heart disease
ChIP-seq	Chromatin immunoprecipitation sequencing
c-KIT	Tyrosine-protein kinase KIT
CM	Cardiomyocyte
CNTN2	Contactin-2
CP	Cardiac progenitor
CX40	Connexin 40
CYP26A1	Cytochrome P450 26A1
DART	Developmental and reproductive toxicity
E10	Embryonic day 10
EB	Embryoid body
EDN1	Endothelin 1
ERBB2	erb-b2 receptor tyrosine kinase 2
EST	Embryonic stem cell test
ET-1	Endothelin-1
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FGF	Fibroblast growth factor
FOXH1	Forkhead Box H1
FOXM1	Forkhead box M1
GATA	GATA transcription factor
GRO-seq	Global run-on sequencing
GSK-3	Glycogen synthase kinase 3
HAND	Heart and neural crest derivatives expressed
HCN	Hyperpolarization activated cyclic nucleotide gated potassium channel
HDAC	Histone deacetylase
hESC	Human embryonic stem cell
HEY2	hes related family basic helix-loop-helix transcription factor with YPRW motif 2
hIPSC	Human induced pluripotent stem cell
HOPX	Homeodomain-Only Protein

HOX	Homeobox
HTS	High-Throughput Screening
IGF	Insulin growth factor
IGF1R	Insulin Like Growth Factor 1 Receptor
INSR	Insulin Receptor
IP3R1	Inositol 1,4,5-triphosphate receptor 1
IRX	Iroquois homeobox
ISH	In situ hybridization
ISL1	Islet-1
ISX	Isoxazole
JNK	c-Jun N-terminal kinase
KAT2	Lysine acetyltransferase 2
KCNA5	Potassium voltage-gated channel subfamily A member 5
KCNJ3	Potassium inwardly rectifying channel subfamily J member 3
KDR	Kinase insert domain receptor
KLF4	Kruppel Like Factor 4
MAPK	Mitogen activated protein kinase
MEF2	Myocyte enhancer factor 2
MEIS1	Meis homeobox 1
mESC	Mouse embryonic stem cell
MESP1	Mesoderm posterior basic helix-loop-helix transcription factor 1
MFI	Mean fluorescent intensity
MHC	Myosin heavy chain
MI	Myocardial infarction
MYC	MYC proto-oncogene, basic helix-loop-helix transcription factor
MYL2	Myosin regulatory light chain 2
MYL7	Myosin light chain 7
MYOD	Myoblast determination protein 1
NCX1	Sodium/calcium exchanger gene
NFAT	Nuclear factor of activated T cells

NKX2-5	NK2 homeobox 5
NOTCH1	Notch receptor 1
NPPA	Natriuretic peptide A
NPPB	Natriuretic peptide B
NR2F2	Nuclear receptor subfamily 2 group F member 2
OCT4	Octamer-Binding Protein 4
PA1	Primary pharyngeal arch
PA2	Secondary pharyngeal arch
PFA	Paraformaldehyde
PITX2	Paired like homeodomain 2
PKA	Protein kinase A
PLN	Phospholamban
PPAR	Peroxisome proliferator-activated receptor
PPP1R10	Protein Phosphatase 1 Regulatory Subunit 10
proCCK	procholecystokinin
PSC	Pluripotent stem cell
p-TEFB	Positive transcription elongation factor
qRT-PCR	Quantitative real-time polymerase chain reaction
RALDH2	Aldehyde dehydrogenase family 1, subfamily A2
RAR	Retinoic acid receptor
RXR	Retinoid x receptor
RYR2	Ryanodine receptor 2
SALV	Salvador
SCN	Sodium voltage-gated channel
SERCA	Sarco/endoplasmic reticulum Calcium-ATPase
SHF	Second heart field
SHZ	Sulfonyl-hydrazone
SLN	Sarcolipin
SMyHC3	Slow myosin heavy chain 3
SOCE	Store-operated calcium entry

SOX2	Sex-Determining Region Y-Box 2
SRF	Serum response factor
STIM1	Stromal interaction molecule 1
TALEN	Transcription activator-like effector nuclease
TBX	T-box transcription factor
TCF4	Transcription factor 4
TET	Ten-Eleven translocation family protein
TF	Transcription factor
TGFβ	Transforming growth factor β
TGFBR	Transforming growth factor beta receptor
TNNT2	Troponin T, Cardiac Muscle
T-tubules	Transverse tubules
TSS	Transcriptional start site
VEGF	Vascular endothelial growth factor
venGFP	Ventricular-specific (anterior) fluorescent reporter
YAP1	Yes1 Associated Protein 1
ZNF281	Zinc finger protein 281

1. Introduction

The formation of the heart is governed by upstream signalling pathways which activate downstream tissue-specific gene regulatory networks controlling cell identity. These networks regulate the proliferation and differentiation of multipotent cardiac progenitors into cardiomyocyte subtypes, and these specialized cells adapt specific features characteristic of atrial and ventricular cardiac chambers. An in depth understanding of this process holds promise for understanding the pathology of congenital heart diseases, a significant societal burden affecting around 1% of live births (Donofrio et al., 2014; Moons et al., 2009). Furthermore, understanding the logic of building the heart might lead to rational solutions to re-build the heart in the aftermath of MI, characterized by the loss of around 1 billion cardiomyocytes (Sadek & Olson, 2020). Thus, there is a great impetus to understand the molecular mechanisms of cardiomyocyte subtype differentiation, the process underlying the formation of cells comprising functional cardiac chambers. Furthermore, there is great value in understanding how chemical compounds affect these subtype-specific differentiation mechanisms, with the hope of improving the identification of teratogens and potentially allowing for the development of regenerative drugs and cell therapies.

In order to systematically study the differentiation mechanisms of cardiomyocyte subtypes, cell-based models are needed which allow chemical and genetic perturbation of cardiomyocyte subtype specification in a tractable system. In this thesis, I present work developing a stem cell-based model of cardiomyocyte subtype specification. To this end, genome editing was used to mark early atrial and ventricular lineages, respectively (Study I). Detailed validation of this cell model included both *in vivo* and *in vitro* characterization of reporter expression. This study illustrated the effects of retinoids, known teratogens and modulators of anterior-posterior patterning, on atrial-ventricular specification of pluripotent stem cells. In studies II and III, this model was used to analyze effects of candidate differentiation modulators. Furthermore, the differentiation assay developed in study I holds promise for the rapid identification of teratogenic compounds, a necessity within drug and chemical industries.

In addition to well-known chemical modulators of developmental processes explored in Study I, novel GATA-targeted compounds were tested for their effects on atrial and ventricular differentiation in Study II. These compounds had been previously shown to impede the interaction of transcription factors GATA4 and NKX2-5, known master regulators of both heart formation and heart failure. This study led to the identification of unique compounds promoting the activation of atrial and ventricular reporters, respectively. Effects of these compounds on cardiac differentiation were further characterized by qRT-PCR and immunoblotting. Additionally, a specific subclass of compounds indicated the relationship between an acetyl-lysine subdomain and differentiation induction, suggesting the role of bromodomain-containing proteins, which bind acetylated lysines.

Delineation of cardiomyocyte subtype specification and resulting phenotypes requires a more detailed understanding of molecular markers of those cell types, in addition to the nature of coordinated action of developmental transcription factors. Peptides expressed by the heart are important markers of heart failure and in some cases are also determinants of disease outcome. In Study III of the thesis, the cardiac expression of the neuropeptide procholecystokinin was characterized in developmental, neonatal, and adult stages. Furthermore, core cardiac transcription factors T-Box transcription factor 5 (TBX5) and myosin enhancer factor 2c (MEF2C) were identified as transcriptional regulators of procholecystokinin expression. The effects of CCK-8 peptide on differentiation mechanisms were also explored using the differentiation assays developed in Study I.

Before presentation and analysis of original findings obtained from these studies, I will provide an overview of the molecular dynamics of cardiac chamber formation based on several decades of published loss- and gain-of-function studies, in addition to the roles of these developmental pathways in adult heart homeostasis and disease. Furthermore, I will present published research on chemical modulation of some of these targets in the context of both teratogenic modulation of developmental processes and adult heart disease phenotypes.

2 Review of the literature

2.1 Formation of the four-chambered heart and chamber-specific manifestations of congenital and adult heart diseases

2.1.1 The function of the adult heart and the necessity for specialized cardiomyocyte subtypes and defined chambers

The heart is singular in function, responsible for pumping nutrient- and oxygen-rich blood throughout the organism. However, this singularity belies an underlying structural, molecular, and electrical complexity which must be tightly controlled in order to maintain rhythmic contractility and eject sufficient amounts of oxygenated blood (Bootman et al., 2006). To accomplish this, the four-chambered mammalian heart is composed of an array of highly specialized cell types comprising a finely patterned and morphologically asymmetric organ (Desgrange et al., 2018; Litviňuková et al., 2020). This organ has stark differences between the atria and ventricles, as well as between the right and left sides of the heart. Additionally, the heart contains specialized cellular wiring for transmission of the electrical impulses controlling beating, called the conduction system. The necessity of structural heterogeneity can be explained by several physiological demands of the beating heart: 1) the electrical impulse which begins the heart beat should only occur in the sinoatrial node of the right atria and must be transmitted sequentially to the rest of the heart via a specialized conduction system (Bootman et al., 2006); 2) atrial contraction and relaxation precede the onset of ventricular contraction (Bootman et al., 2006); 3) the ventricles must pump blood more strongly than the atria as they are responsible for pushing blood to tissues outside of the heart (Bootman et al., 2006); 4) the relative force of atrial contraction must increase during exercise in order to refill the ventricles (Bootman et al., 2006); and 5) the left ventricle (itself supplied with blood by the coronary artery) is necessarily more muscular than the right ventricle (left-right asymmetry) in order to pump blood to all tissues of the body (systemic circulation), rather than only to the lungs (pulmonary circulation) (Desgrange et al., 2018). Formation of this structurally complex and molecularly heterogeneous organ requires the proper execution of a complex series of events during embryogenesis (Moorman et al., 2003). These instructions are encoded in the genome and carried out by a large number of molecular entities, many of which are also integral to adult heart function and disease (Xin, Olson et al., 2013). Insight into the molecular underpinnings of both the formation and function of the chambers of the heart has led to an improved understanding of both congenital and adult heart disease. Similarly, exploration of congenital and adult heart diseases has given insight into basic mechanisms underlying heart formation and could lead to new strategies for replacing cells lost after MI (Xin, Olson et al., 2013). It is thus worthwhile to examine the developmental origins, signals and gene regulatory networks governing the formation of specialized cells in the heart, as well as the molecular architecture which gives rise to their unique function. Finally, chemical manipulation of these pathways reveals teratogenic side effects of pharmacological drugs, in addition to potentially identifying regenerative mechanisms of action of targeted therapies for the treatment of adult diseases.

2.1.2 A brief morphological description of cardiogenesis

An astonishingly complex series of events encompass the formation of the adult heart from a sheet of progenitor cells in the early embryo, and these are orchestrated by transcription factor networks (Figure 1a-b). The earliest cells giving rise to the heart appear during gastrulation, an invagination of embryonic tissue leading to formation of the three germ layers which give rise to all organs: the ectoderm, endoderm, and mesoderm (human: around 3 weeks, mouse around embryonic day 7) (Moorman et al., 2003). From the mesodermal layer is formed the cardiac crescent, and this structure contains the promyocardial cells which begin to express sarcomeric markers and initiate the Ca^{2+} activity which will underlie the first heart beat (Moorman et al., 2003; Tyser et al., 2016). The primitive myocardial cells surround primitive endocardial cells to form a linear heart tube comprised of an outflow tract, primitive ventricle, venous pole (primitive atria), and sinus horns, and this tube-like structure begins to beat spontaneously (mouse E8.0) (Moorman et al., 2003; Tyser et al., 2016). This initial heart tube is derived from first heart field cells of the cardiac crescent, whereas a second heart field of undifferentiated cells later migrate into the primordial heart and contribute to the atria, outflow tract, and the entirety of the right ventricle (Moorman et al., 2003; Zaffran et al., 2004). This linear tube then bends to the right, and at this looping heart stage the primitive atria and ventricles are

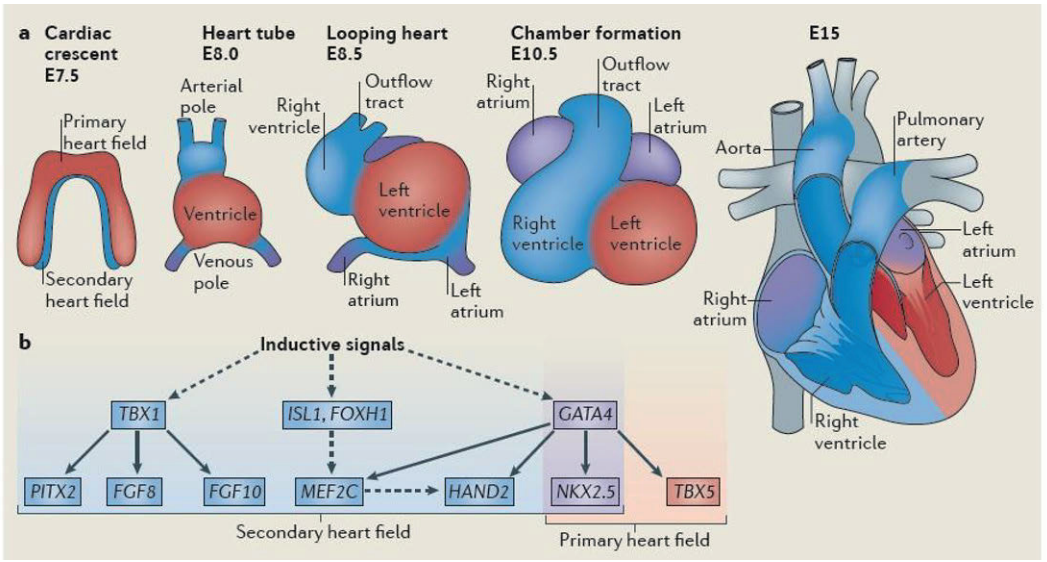

Figure 1. Overview of cardiogenesis. **a** The cardiac crescent consists of two progenitor populations, the primary and secondary heart fields, which fuse at the midline to form the spontaneously beating linear heart tube. The linear heart tube loops to the right, leading to formation of the primitive four-chambered heart. The left ventricle, pictured in red, is derived exclusively from primary heart field cells, whereas the right ventricle is derived from secondary heart field cells. Atrial tissue is derived from both primary and secondary heart field cells. Later stages of development include formation of the great arteries, conduction system, septa, and trabeculated myocardium. **b** The core cardiac transcription factor network consists of GATA4, MEF2C, NKX2-5 and TBX5. TBX5 is specific to primary heart field progenitors and derived tissues, whereas MEF2C is specific to secondary heart field and those derived tissues. E = mouse embryonic day. With permission from Xin, Olson et al., 2013. T-box transcription factor 1 (TBX1), Islet-1 (ISL1), Forkhead Box H1 (FOXH1), GATA transcription factor 4 (GATA4), Paired like homeodomain 2 (PITX2), Fibroblast growth factor 8 (FGF8), Fibroblast growth factor 10 (FGF10), Myocyte enhancer factor 2c (MEF2C), Heart and neural crest derivatives expressed (HAND2), NK2 homeobox 5(NKX2.5), T-box transcription factor 5 (TBX5).

noticeably separated by the atrioventricular canal (Moorman et al., 2003). Also, at this stage chamber-specific gene expression delineating the atria and ventricles is first apparent (O'Brien et al., 1993; Xavier-Neto et al., 1999). Blood begins to flow into the unseptated atria and through the primitive ventricles, and thereafter exits via the outflow tract (Moorman et al., 2003). Also, a primitive interventricular septum dividing the right and left ventricles is visible at this stage (Moorman et al., 2003). The atria grow in concert with the formation of the lungs and the connection of the lungs to the left part of the common, unseptated atrium via the pulmonary vein (Moorman et al., 2003). The ventricles concurrently grow and are visually characterized by rough invaginations called trabeculae, in contrast to the atria which are untrabeculated and smooth (Moorman et al., 2003). Growth of both the atria and ventricles is driven by hyperproliferative cardiomyocytes expressing chamber-specific markers, and the proliferative rate gradually decreases during the remainder of fetal development and early postnatal life (Sedmera & Thompson, 2011). A mostly muscular wall fully forms between the two ventricles (interventricular septum) and between the atria and ventricles (atrioventricular septum) (Anderson et al., 2003). These events complete the formation of the primitive, four chambered heart, a remarkable feat of mammalian evolution.

2.1.3 When morphogenesis goes awry – congenital heart disease

Owing to the complexity of cardiac formation, it is unsurprising that this process is highly susceptible to both genetic and environmental perturbation. Strikingly, congenital heart diseases (CHDs) have been estimated to occur in 0.6-1.2% of live births, and this increases to 8.6% if stillborn births are included (Donofrio et al., 2014; Moons et al., 2009). Risk factors for CHDs include underlying genetic or metabolic conditions, as well as exposure to environmental contamination and certain drugs (Donofrio et al., 2014). Genetic mutations appear to underlie a large fraction of congenital heart diseases. For instance, after the Fukushima nuclear accident in Japan, the number of corrective surgeries for congenital heart disease increased by 15%, ostensibly due to the occurrence of *de novo* genetic mutations induced by radiation exposure (Murase et al., 2019). Maternal illnesses such as diabetes, viral infections, and phenylketonuria were also shown to increase the likelihood of congenital heart diseases (Jenkins et al., 2007). Additionally, maternal exposure to teratogenic drugs, organic solvents, or other environmental pollutants have been shown to induce cardiac malformations (Jenkins et al., 2007). Understanding the molecular mechanisms of both genetic and environmental perturbation of cardiogenesis can provide insight into development of the heart and congenital disease mechanisms.

The panoply of congenital heart diseases observed in neonates mostly includes those affecting specific structures and/or chambers of the heart, whereas gross malformation of the organ typically results in embryonic/fetal lethality (described in later sections). Congenital heart diseases are generally divided into three categories 1) cyanotic heart diseases in which oxygenated and deoxygenated blood mix 2) left-side obstruction defects and 3) septation defects (Bruneau, 2008). Clinical presentation is often characterized by multiple traits, and these diseases often display defects in arterial, venous, or valve formation which are amenable to surgical correction (Bruneau, 2008; Donofrio et al., 2014). Though these innovative surgeries now allow survival into adulthood, surviving patients are often more at risk for adult-onset diseases such as arrhythmias, heart failure, and myocardial infarction, possibly due to persistence of structural abnormalities or underlying disease mutations (Christophersen & Ellinor, 2016; Donofrio et al., 2014; Mueller et al., 2020; Olsen et al., 2017; Walsh & Cecchin, 2007). Indeed, these cases might represent opportunities to develop targeted therapies for patients containing specific mutations.

2.1.4 Cardiomyocyte-subtype specific manifestations of adult-onset cardiovascular diseases

Similar to congenital heart diseases, adult cardiac diseases are often due to dysfunction in specific subtypes and subregions of the heart: most notably atrial cardiomyocytes, ventricular cardiomyocytes, and conduction system cells (Figure 2). For instance, atrial fibrillation is due to aberrant excitation of atrial cardiomyocytes and the disturbance of synchronized contraction of the heart (Feghaly et al., 2018). This is the most commonly observed arrhythmia, and has been reported to increase the risk of

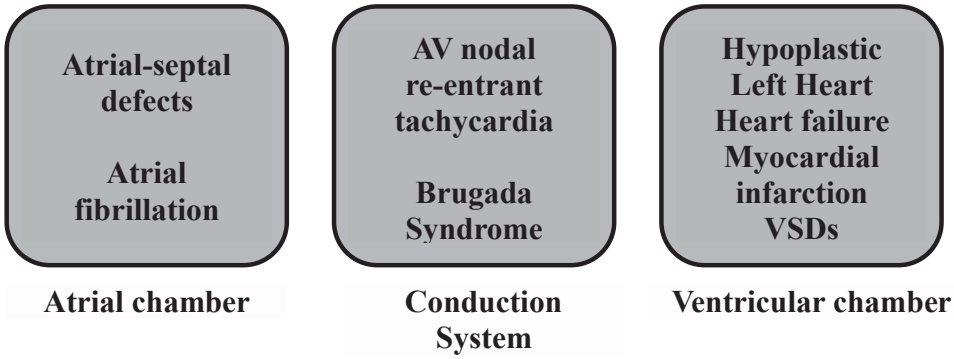

Figure 2. Chamber-specific manifestations of congenital and adult heart diseases necessitating investigation into chamber-specific cardiomyocyte biology. Collectively, cardiovascular diseases are the global leading cause of deaths (GBD 2017, 2018). Curative therapies are still lacking. Ventricular-septal defects (VSDs).

stroke five-fold, amplifying the severe economic and societal burden of this disease (Feghaly et al., 2018; Wolf et al., 1991). Additionally, atrial fibrillation frequently occurs in patients with heart failure, further complicating its treatment (Li et al., 1999; Maisel et al., 2003). Interestingly, disease risk loci for atrial fibrillation include many genes which are involved in formation of the heart (cardiogenesis), as well as those with distinct chamber- and subtype-specific expression patterns (Christophersen & Ellinor, 2016; Feghaly et al., 2018). Thus, atrial fibrillation represents a cardiomyocyte-subtype specific disease, and investigation of the biology of atrial development might lead to improved insights into disease etiology. In addition to atrial fibrillation, other electrical disturbances of the heart have cardiomyocyte subtype-specific manifestations. These include diseases manifested in the atrioventricular canal (AV nodal reentrant tachycardia), ventricle (recurrent ventricular tachycardia), and bundle branch of the right ventricle (Brugada syndrome), specifically (Saffitz & Corradi, 2016). A full understanding of the molecular mechanisms underlying electrical disorders thus requires specific study of these anatomical structures and the molecular markers which can be used to characterize them.

Myocardial infarction (MI) results in the rapid loss of around one billion cardiomyocytes which are not subsequently replaced, and this results in more deaths globally than any other single cause (Sadek & Olson, 2020). Post- MI pharmacological treatment can slow the progression to heart failure, but this does not address the central problem of the loss of cardiomyocytes (Sadek & Olson, 2020). Though infarctions in atrial chambers do occur, myocardial infarctions predominantly affect the left ventricle, necessitating the development of methods to replace lost cardiomyocytes with cells possessing left ventricular identity (Lu et al., 2016). Despite many claims of native myocardial progenitors in the adult heart, in addition to other reports that hematopoetic stem cells can differentiate into *bona fide* cardiomyocytes, a consensus has recently emerged that these studies were not reliable, and that there is in fact no endogenous cardiomyocyte progenitor in the adult heart

(Eschenhagen et al., 2017; Sadek & Olson, 2020). Thus, the current strategies being most actively explored for replacement of lost cardiomyocytes can be divided into three categories: 1) transplantation of pluripotent stem cell-derived cardiomyocytes into the infarcted heart; 2) reprogramming of non-myocytes in the heart to the cardiomyocyte fate using viral-mediated overexpression of developmental factors; and 3) induction of proliferation of existing cardiomyocytes in the heart (Sadek & Olson, 2020). Intriguingly, these strategies all have strong foundations in embryology, and the full characterization of the molecular mechanisms governing the formation of cardiomyocytes from undifferentiated pluripotent stem cells (PSCs) might hold the clues for the development of regenerative therapies (Yi et al., 2010). Furthermore, the understanding of cell fate commitment and maturation of not just cardiomyocytes, but cardiomyocyte subtypes might allow for refinement of these strategies.

2.1.5 Endogenous and induced cardiac regeneration of the ventricle

Nearly fifty years ago, it was discovered that the ventricles of lower vertebrates such as salamanders and newts were capable of regeneration of lost myocardium following injury (Becker et al., 1974; Oberpriller & Oberpriller, 1971). This was later shown to be due to de-differentiation, proliferation, and subsequent re-differentiation of pre-existing cardiomyocytes (Jopling et al., 2010; Wang et al., 2017). Surprisingly, it was also shown that reprogramming of atrial cardiomyocytes to ventricular cardiomyocytes occurred during regeneration of the zebrafish ventricle (Zhang et al., 2013). In recent years, it was revealed that the hearts of neonatal mice are capable of significant regeneration upon myocardial insult, raising hopes that this could also be feasible in larger mammals, such as humans (Porrello et al., 2011). Interestingly, follow-up studies have revealed some of the mechanisms of this process. For instance, deletion of monocytes/macrophages impeded cardiac regeneration, suggesting that the immune response is necessary for regeneration to occur (Aurora et al., 2014). Additionally, limited adult mouse heart regeneration is correlated with the presence of mononuclear cardiomyocytes, which themselves show variable levels across different inbred mouse strains, suggesting that genetically-encoded binucleation patterns also influence regenerative capacity (Patterson et al., 2017). Though it is unknown to what degree mononuclear cardiomyocytes vary across human populations, there is now conclusive evidence for the existence of cardiomyocyte proliferation in the adult human heart. In a landmark study, use of carbon-14 integrated into the hearts of the global human population during above-ground nuclear tests of the Cold war period indicated that cardiomyocyte proliferation occurs at around 1% per year (Bergmann et al., 2009). A follow-up study revealed that cardiomyocyte proliferation is highest during childhood and subsequently decreases with age (Bergmann et al., 2015). Therefore, recent studies provide evidence for the pursuit of cardiomyocyte proliferation as a therapeutic modality.

Despite the promise of cardiac regeneration mediated by stimulation of endogenous human cardiomyocyte proliferation, questions still remain as to adverse effects of excess cardiomyocyte proliferation on the function of the heart. When the SV40 T antigen was expressed under the control of the Natriuretic peptide A (Nppa) promoter, resulting in unrestrained proliferation of the atrium, it induced an increase in the size of the right atrium, arrhythmias, and death (Field, 1988). Though this was based on overexpression of a viral protein, recent studies have provided similar findings in a more physiologically relevant context. In adult mouse hearts post-myocardial infarction, injection of the growth factor neuregulin induced cardiomyocyte proliferation and myocardial regeneration, suggesting that it could be used as a therapeutic agent (Bersell et al., 2009). In a later study, the neuregulin co-receptor erb-b2 receptor tyrosine kinase 2 (ERBB2) was shown to be downregulated one week after birth, and overexpression during juvenile and adult stages resulted in cardiomyocyte

(CM) proliferation, suggesting the neuregulin pathway might be a major factor determining the capacity for neonatal heart regeneration (D'Uva et al., 2015). However, though ERBB2 extended the regenerative window of neonatal mice, it also resulted in cardiomegaly (an oversized heart), pointing towards potential risks of this approach (D'Uva et al., 2015). Similarly, recent studies achieving micro-RNA mediated cardiomyocyte proliferation in the adult pig heart resulted in an abundance of undifferentiated myoblasts, arrhythmias, and sudden death (Gabisonia et al., 2019). These failures are likely due to the inherent nature of adult cardiomyocyte proliferation, which involves a three-step process of de-differentiation, proliferation, and re-differentaition (Wang et al., 2017). Thus, the induction of not only de-differentiation, but also re-differentiation of pre-existing cardiomyocytes might be needed to achieve therapeutic cardiac regeneration. Furthermore, it is unknown if the induction of cell proliferation modifies the subtype identity or maturity of chamber-specific cardiomyocytes.

2.1.6 Survivors of congenital heart disease: a high risk population for development of adult heart disease

The development of surgical procedures performed in the neonate (or even *in utero*) have led to the survival of infants with congenital heart disease into adolescence and adulthood. However, genetic (or epigenetic) etiologies of malformations are not repaired, and alterations in gene regulatory networks can cause problems in adulthood. Strikingly, atrial arrhythmias occur in 15% of adults with congenital heart disease and in greater than 50% of adults with severe congenital heart disease who reach the age of 65, suggesting shared molecular pathways exist which govern both formation of the heart and atrial rhythm (Bouchardy et al., 2009). Large-scale studies also suggest that similar genetic pathways underlie CHDs and severity of myocardial infarction. In a Danish study, CHD patients were more likely to suffer from a myocardial infarction and had increased 30-day mortality versus patients who did not previously suffer from a CHD (Olsen et al., 2017). To date, there are no reports of tailored drug regimens for patients suffering from congenital heart disease due to mutations in known disease-driver genes.

2.1.7 *In vivo* and *in vitro* methods for understanding cardiovascular lineage diversification, gene function, and pharmacology

The development of *in vivo* and *in vitro* methods for studying cell fate commitment has led to an abundance of knowledge regarding cardiovascular lineage diversification. Cre-LoxP based systems allow for deletion of genomic regions in cells expressing a specific marker protein, allowing the study of gene function and cell fate commitment in defined cell lineages at specific stages of development (Gu et al., 1993; Mao et al., 1999). This technology has been instrumental to the study of cardiovascular lineage diversification and gene function during embryogenesis, in addition to the validation of therapeutically relevant targets in the adult heart. A summary of Cre mouse lines used for deletion in specific regions of the heart is shown in Table 1.

In addition to *in vivo* Cre-LoxP systems, the simultaneous development of PSC technology has led to an abundance of studies in which *in vitro* PSC differentiation has been used to study cardiovascular lineage diversification. Both *in vivo* and *in vitro* methods were dependent on the landmark discovery that PSCs could be isolated from early blastocyst-stage mouse embryos and maintained in the pluripotent state, representing a method later used for generation of genetically modified mice and the study of early embryological processes (Evans & Kaufman, 1981; Robertson et al., 1986; Thomas & Capecchi, 1986; Williams et al., 1988). In the absence of conditions for maintaining pluripotency *in vitro*, PSCs were shown to form embryoid bodies and to differentiate into cells of the three germ

layers (Doetschman et al., 1985). These included mesoderm-derived cardiomyocytes which were easily discernable by the formation of spontaneously beating clusters (Doetschman et al., 1985).

mouse line	cell lineage (s)	citations
cGata6-Cre	Atrioventricular canal	Davis et al., 2001
CCS-LacZ	Conduction system cells	Rentschler et al., 2001
Cntn2-EGFP	Conduction system cells	Pallante et al., 2010
Hcn4-Cre	First heart field mesodermal progenitors, conduction system cells	Liang et al., 2013
Isl1-Cre	Second heart field mesodermal progenitors which give rise to right ventricle and parts of atria	Cai et al., 2003; Srinivas et al., 2001
Mesp1-Cre	Earliest marker of cardiac mesoderm	Saga et al., 1999
Mhc-Cre	All cardiomyocytes	Agah et al., 1997
Myl2-Cre	Ventricular cardiomyocytes	Chen et al., 1998
Nkx2-5-Cre	Cardiac progenitor cells, embryonic cardiomyocytes	Moses et al., 2001; Stanley et al., 2002
Sln-Cre	Atrial cardiomyocytes	Shimura et al., 2016

Table 1. Mouse lines used for the study of cardiovascular lineage diversification and gene function. Cre lines allow the study of cell contribution to anatomical structures in addition to the specific deletion of genes of interest within those cell lineages. GATA transcription factor 6 (Gata6), Cardiac conduction system (CCS), Contactin-2 (Cntn2), Hyperpolarization activated cyclic nucleotide gated potassium channel 4 (Hcn4), Islet-1 (Isl1), Mesoderm posterior basic helix-loop-helix transcription factor 1 (Mesp1), Myosin heavy chain (Mhc), Myosin light chain 2 (Myl2), NK2 homeobox 5 (Nkx2-5), Sarcolipin (Sln).

The utility of PSCs in pharmacological and toxicological studies was quickly realized. PSC-derived cardiomyocytes were shown to respond to chemical chonotropic modulators, establishing them as an *in vitro* model of cardiotoxicity (Wobus et al., 1991). Furthermore, the differentiation of PSCs to cardiomyocytes was established as a model of reproductive toxicity and formalized as the Embryonic Stem Cell Test (EST) (Scholz et al., 1999). Thus, pluripotent stem cell technology is applicable to the study of pharmacological induction of both congenital and adult heart diseases. Though early work was restricted to mouse PSCs, the isolation of pluripotent stem cells from human blastocysts (human embryonic stem cells, hESCs), and later the induction of pluripotency in human dermal fibroblasts (human induced pluripotent stem cells, hIPSCs) allowed for the study of human cardiomyocyte

differentiation and even patient-specific cardiomyocytes (Takahashi et al., 2007; Thomson et al., 1999).

In the past decade, *in vitro* methods for differentiation of both mouse and human PSCs have been improved regarding their efficiency, subtype-specificity, and maturity. For instance, modulation of developmental signaling pathways and metabolic selection have led to differentiation protocols producing nearly pure cultures of beating cardiomyocytes (Kattman et al., 2011; Lian et al., 2012; Tohyama et al., 2013). However, differentiation of cells to a specific cardiomyocyte subtype lineage is also important, as early patch clamp studies demonstrated that spontaneous differentiation of mouse PSCs gave rise to cardiomyocytes with action potential shapes representing ventricular, atrial, and conduction system cells (Maltsev et al., 1993). This appears to depend on the protocol being used, as more efficient directed differentiation protocols give rise to mostly ventricular cardiomyocytes, and only by further modulation of retinoid signaling have defined protocols to the atrial lineage been developed (Lee et al., 2017). Impressively, further improvements in maturation and three-dimensional organoid culture have led to the establishment of human PSC-derived atrial and ventricular drug screening platforms (Goldfracht et al., 2020; Zhao et al., 2019). New differentiation protocols might also improve the prospects for cardiac regeneration via cell therapy. Exogenous delivery of PSC-derived cardiomyocytes has been shown to regenerate both small and large mammalian hearts, but this strategy also resulted in the generation of dangerous arrhythmias (Laflamme et al., 2007; Liu et al., 2018; Romagnuolo et al., 2019). Future efforts might be focused on improved protocols for cardiomyocyte differentiation and/or combined delivery of PSC-derived cardiomyocytes and anti-arrhythmic drugs. Collectively, these studies demonstrate the importance of improvement in stem cell differentiation to the cardiomyocyte fate.

2.1.8 Multipotent cardiac progenitor cells in the developing and adult heart

Lineage tracing studies in the mouse, in addition to *in vitro* studies of the differentiation of pluripotent stem cells to the cardiomyocyte fate, have led to the identification of molecular markers of multipotent CPs and transient intermediates. These markers have been critical to the understanding of cardiovascular development and the application of these principles to regenerative medicine. Additionally, progenitor populations expressing some of these markers have been expandable *in vitro* and thus might serve as cell sources to be injected into the infarcted heart. A summary of these cell populations is shown in Table 2.

Embryonic, multipotent cell populations are capable of giving rise to different cell types in the heart, namely cardiomyocytes, smooth muscle cells, and endothelial cells. Cell markers of these progenitors include tyrosine protein kinase KIT (c-Kit), kinase insert domain receptor (Kdr), and Islet-1 (Isl1) (Kattman et al., 2006; Moretti et al., 2006, Wu et al., 2006). The ability to expand some of these cell populations *in vitro* has further increased their suitability as models of differentiation and regenerative therapies. Furthermore, these markers serve as useful signposts during the optimization of *in vitro* differentiation protocols. They also give indications of the cellular mechanisms of growth and patterning of the early heart. Interestingly, the secondary pharyngeal arch (PA2) of embryonic day 8-10 (E8-10) embryos contains undifferentiated cardiac progenitors, and these may be cultured *ex vivo* in order to model the migration and differentiation of cardiomyocytes from Isl1+ cells (Andersen & Kwon, 2015; Shenje et al., 2014). Thus, embryonic progenitor populations can provide insight into mechanisms of cardiogenesis, in addition to serving as cell sources for *in vitro* modelling.

Though the existence of embryonic cardiomyocyte progenitors is widely accepted, earlier reports of cardiomyocyte progenitor cell activity in the adult heart have recently been refuted (Maliken et al., 2018; Neidig et al., 2018). However, these studies have revealed that active cardiac progenitors do

Progenitor cell marker	Cardiovascular Lineages in embryo	Citations
c-Kit+ Nkx2-5+	cardiomyocytes, smooth muscle cells	Wu et al., 2006
Kdr+	cardiomyocytes, smooth muscle cells, endothelial cells	Kattman et al., 2006
Hopx+	cardiomyocytes	Jain et al., 2015
Isl1+	cardiomyocytes, smooth muscle cells, endothelial cells	Moretti et al., 2006
Mesp1+	cardiomyocytes, smooth muscle cells, endothelial cells	Bondue et al., 2011

Table 2. Markers of cardiac progenitor cells with varying degrees of potency. These markers allow isolation and expansion of progenitor cells *in vitro* and may have utility in the development of regenerative therapies. They also serve as signposts for the development of differentiation protocols. Tyrosine-protein kinase KIT (c-Kit), NK2 homeobox 5 (Nkx2-5), Kinase insert domain receptor (Kdr), Homeodomain-Only protein (Hopx), Islet-1 (Isl1), Mesoderm posterior basic helix-loop-helix transcription factor 1 (Mesp1).

indeed give rise to endothelial cells in the heart, and it is unknown to what extent their cardiogenic potential could be induced by therapeutic agents. Furthermore, it is unknown whether *ex vivo* culture conditions might change their epigenetic state to allow them to have increased cardiogenic potential. A recent study on a new progenitor population might shed light on some of these controversial findings. Lineage tracing studies indicated that a Twist2+ progenitor population in the adult heart gave rise to cardiomyocytes, endothelial cells, and fibroblasts; though most contribution to cardiomyocytes was via cell fusion with pre-existing cardiomyocytes (Min et al., 2018). Thus, the cell fusion phenomenon described therein might explain the contribution of stem cells to cardiomyocytes during lineage tracing studies in the adult heart. Interestingly, adult cardiomyocytes have been shown to undergo mitosis after cell fusion with proliferating non-myocytes (Matsuura et al., 2004). It is unknown to what degree fusion of progenitor cells with differentiated cardiomyocytes might positively or negatively affect their organ-level function and regenerative potential. Furthermore, it is unknown if this process could be stimulated with drugs.

2.2 Upstream signaling pathways underlying cardiogenesis and their role in congenital and adult heart diseases

Cardiogenesis is largely driven by secreted proteins which form localized gradients within undifferentiated cell populations to activate cell differentiation programs. Oftentimes, the same signaling pathways display distinct spatial and temporal deployment to form different tissues in different parts of the embryo. Pluripotent stem cells and progenitors receive differentiation signals, and this leads to activation of gene regulatory networks governing cell fate, ultimately leading to the activation of cell-type specific structural genes in defined subtypes (Figure 3). Mouse genetics, allowing temporal and spatial depletion of signaling pathway components, has led to a precise understanding of the upstream signals regulating cardiogenesis and the role of these signals in adult heart function. A subset of these studies is described in the following sections. Despite the abundance

of significant work on signaling pathways in other model organisms, this section will be focused on studies in mice. Relevant mouse loss- and gain-of-function studies are summarized in Table 3.

2.2.1 Wnt signaling

Wnts are secreted proteins which act on nearby cells via the activation of the downstream transcriptional effector β-catenin (canonical) or increases in intracellular Ca^{2+} (non-canonical) (Slusarski et al., 1997; Wiese et al., 2018). Though important to the formation of many tissues, deletion of Wnt signaling components in cardiovascular lineages has demonstrated their role in heart formation, specifically. For instance, deletion of β-catenin in early mesodermal progenitors marked by mesoderm posterior basic helix-loop-helix transcription factor 1 (Mesp1) resulted in failure to form the second heart field-derived right ventricle, in addition to disruption of cardiac looping (Klaus et al., 2007). Deletion of β-catenin in Isl1+ second heart field progenitors demonstrated that Wnt

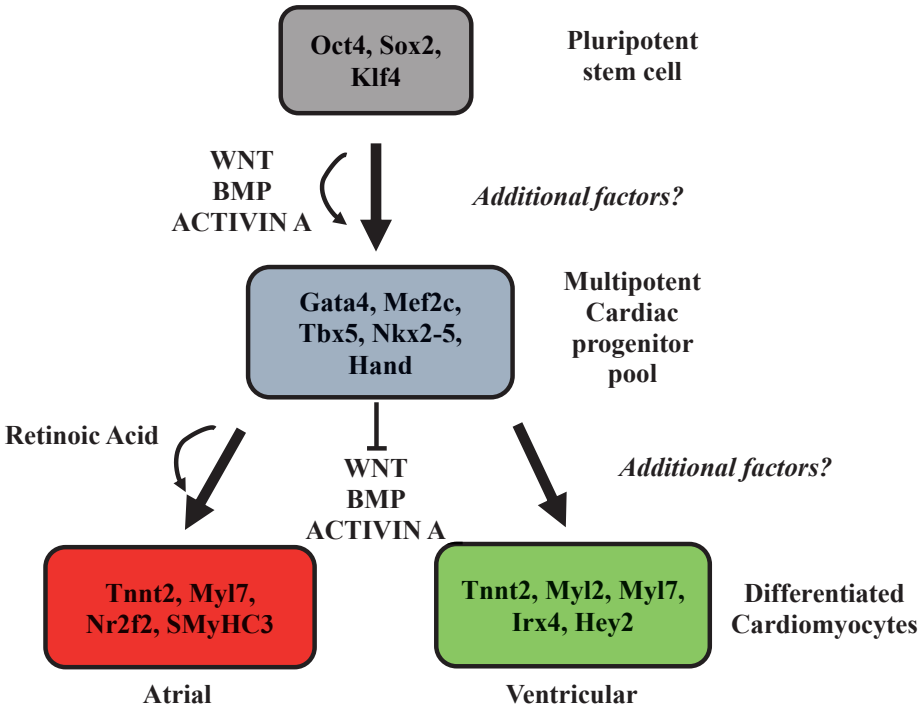

Figure 3. Transcription factors, signaling pathways, and cell-type specific markers during the differentiation of pluripotent stem cells to atrial and ventricular cardiomyocytes. Signalling pathways promoting the specification of cardiac progenitors from pluripotent stem cells must then be repressed in order for the differentiation of functional cardiomyocytes to occur. Retinoic acid signaling is a key driver of differentiation to atrial cardiomyocytes. Myl2, a ventricle-specific marker may be used to identify ventricular cardiomyocytes. Octamer-Binding Protein 4 (Oct4), Sex-Determining Region Y-Box 2 (Sox2), Kruppel Like Factor 4 (Klf4), Bone Morphogenetic Protein (BMP), GATA transcription factor 4 (Gata4), Myocyte enhancer factor 2c (Mef2c), T-box transcription factor 5 (Tbx5), NK2 homeobox 5 (Nkx2-5), Heart and neural crest derivatives expressed (Hand), Troponin T, Cardiac Muscle (Tnnt2), Myosin Light Chain 7 (Myl7), Nuclear receptor subfamily 2 group F member 2 (Nr2f2), Slow myosin heavy chain 3 (SMyHC3), Myosin light chain 2 (Myl2), Iroquois homeobox 4 (Irx4), hes related family basic helix-loop-helix transcription factor with YPRW motif 2 (Hey2)

gene	upstream signalling pathway	Role in multipotent cardiac progenitors/early cardiogenesis	Role in differentiated cardiomyocytes/late cardiogenesis	Role in postnatal heart	citations
β-catenin	Wnt	Deletion in Mesp1 lineage leads to failure of heart looping and lack of right ventricle; Deletion in Isl1 (SHF) lineage impedes expansion of SHF progenitors and formation of the right ventricle.	Post-mesoderm deletion impedes proliferation of ventricular cardiomyocytes, formation of atrioventricular canal.	Cardiomyocyte-specific deletion improved cardiac function following MI	Gillers et al., 2015; Klaus et al., 2007; Kwon et al., 2007; Zelarayán et al., 2008
Bmp2	Bmp	Deletion results in early lethality due to many defects, heart in exocoelomic cavity or lack of heart.	Overexpression in Nkx2-5+ cells leads to increased cardiomyocyte proliferation, reduced cardiomyocyte maturation, and E15.5 death.	-	Prados et al., 2018; Zhang & Bradley, 1996
Bmp4	Bmp	Germline deletion leads to absence of mesoderm and embryonic lethality prior to E9.5.			Winnier et al., 1995
Bmp10	Bmp		Germline deletion leads to reduced proliferation of cardiomyocytes and myocardial thinning.		Chen et al., 2004
BmpR1a	Bmp	Deletion in Mesp1 lineage leads to absence of cardiac crescent and primitive (left) ventricle.	Cardiomyocyte-specific deletion impedes septation and increases apoptosis, E18.5 death. Atrioventricular canal expression is necessary for formation of the atrioventricular valves.	-	Gaussin et al., 2002; Gaussin et al., 2005; Klaus et al., 2007
Cyp26a1/Cyp26c1	Retinoic acid	Combinatorial deletion in zebrafish results in expansion of atrial cardiomyocytes.	-	-	Rydeen & Waxman, 2014

Table 3 (continued on next three pages). Cardiovascular effects of deletion of signalling pathway genes during early/late embryogenesis and adulthood. Functional studies provide evidence for the role of specific signalling pathways in cardiogenesis and adult cardiac homeostasis. Bone morphogenetic protein 2 (Bmp2), Bone morphogenetic protein 4 (Bmp4), Bone morphogenetic protein 10 (Bmp10), Bone morphogenetic protein receptor type 1a (Bmpr1a), Cytochrome P450 26A1 (Cyp26a1), Cytochrome P450 26C1 (Cyp26c1), Endothelin 1 (Edn1), Glycogen synthase kinase 3β (Gsk3β), Insulin Like Growth Factor 1 Receptor (Igf1r), Insulin Receptor (Insr), Notch Receptor 1 (Notch1), Aldehyde dehydrogenase family 1, subfamily A2 (Raldh2), Retinoic acid receptor alpha variant 1 (Rarα1), Retinoic Acid Receptor Beta (Rarβ), Retinoid X Receptor Alpha (Rxrα), Salvador (Salv), Smoothened, Transforming growth factor beta 2 (Tgfβ2), Yes1 Associated Protein 1 (Yap1).

gene	upstream signalling pathway	Role in multipotent cardiac progenitors/early cardiogenesis	Role in differentiated cardiomyocytes/late cardiogenesis	Role in postnatal heart	citations
Edn1	Endothelin	-	Germline deletion leads to outflow tract defects, ventricular-septal defects, aortic arch defects; impeded sympathetic innervation of the heart. Regulates embryonic cardiac contractility.	Cardiomyocyte-specific deletion leads to aging-associated heart failure in mice and premature death. Exogenous treatment causes increased Nppa expression and cellular hypertrophy. Regulates contractility and force generation. Cardiomyocyte overexpression leads to electrical remodeling and heart failure.	Fukuda et al., 1989; Ieda et al., 2004; Ito et al., 1991; Karppinen et al., 2014; Kurihara et al., 1995; Mueller et al., 2011; Piuhala et al., 2003; Yang et al., 2004; Zhao et al, 2006
Gsk3β	Wnt (inhibitor of)	-	Germline deletion leads to increased cardiomyocyte proliferation and myocardial hyperplasia, double outlet right-ventricle, and ventricular-septal defects.	Post MI deletion leads to increased cardiomyocyte proliferation and improved ventricular function. Co-deletion of both Gsk3α/Gsk3β causes cardiomyocyte proliferation and leads to ventricular dysfunction and death. Cardiomyocyte-specific overexpression of active Gsk3β impedes cardiac hypertrophy following pressure overload *in vivo*.	Antos et al., 2002; Kerkela et al., 2008; Woulfe et al., 2010; Zhou et al., 2016
Igf1r/Insr	Insulin	-	Co-deletion in Nkx2-5+ cells leads to decreased proliferation and myocardial wall thinning.	Cardiomyocyte-specific overexpression of Igf1r leads to physiological hypertrophy of the heart and increased systolic function.	Li et al., 2011; McMullen et al., 2004
Notch1	Notch	Deletion in Isl1+ SHF mesodermal progenitors increases Wnt signalling and expansion of progenitors.	Germline deletion leads to defects in ventricular proliferation and trabeculation. Human mutations underlie aortic valve defects.	-	Garg et al., 2005; Grego-Bessa et al., 2007; Kwon et al., 2009
Raldh2	Retinoic acid	Germline deletion leads to failure to undergo cardiac looping, expansion of SHF mesodermal progenitors, lethality at E10.5.	-	-	Niederreither et al., 2001; Ryckebusch et al., 2008

gene	upstream signalling pathway	Role in multipotent cardiac progenitors/early cardiogenesis	Role in differentiated cardiomyocytes/late cardiogenesis	Role in postnatal heart	citations
Rarα1/Rarβ	Retinoic acid	-	Germline deletion impedes proliferation of ventricular cardiomyocytes and ventricular septation, leads to cardiac outflow defects.	Cardiomyocyte-specific deletion of RARα1 leads to impaired diastolic function, downregulation of Serca2, and calcium handling perturbation. Rars are activated post-MI.	Bilbija et al., 2012; Lee et al., 1997; Zhu et al., 2016
Rxrα	Retinoic acid	-	Germline deletion impedes proliferation of ventricular cardiomyocytes and ventricular septation, leads to cardiac outflow defects, leads to continued atrial gene expression in the ventricles.	Overexpression leads to altered myofibrils, metabolic alterations, and dilated cardiomyopathy.	Dyson et al., 1995; Gruber et al., 1996; Subbarayan et al., 2000; Sucov et al., 1994
Salv	Hippo	-	Deletion in Nkx2-5+ cells lead to enlarged hearts, increased proliferation, ventricular-septal defects, upregulation of Wnt signaling.	Knockdown or knockout improves heart function following MI.	Heallen et al., 2011; Leach et al., 2017
Smoothened	Hedgehog signaling	Germline deletion leads to failure of cardiac looping and failed activation of downstream cardiac TFs.	-	Cardiomyocyte-specific deletion in adult rats leads to loss of coronary vasculature and heart failure.	Lavine et al., 2008; Zhang et al., 2001
Tgfβ2	Tgfβ	Germline deletion leads to increased apoptosis, AV canal defects, ventral-septal defects, aortic/branching defects.			Bartram et al., 2001
Yap1	Hippo (inhibited by)	-	Deletion in embryonic cardiomyocytes leads to reduced cardiomyocyte proliferation and myocardial hypoplasia, Overexpression leads to increased proliferation of fetal cardiomyocytes.	Deletion impedes cardiomyocyte proliferation and neonatal heart regeneration. Overexpression leads to increased cardiomyocyte proliferation and promotes heart regeneration and contractility post-MI.	von Gise et al., 2012; Xin et al., 2013

signaling was necessary for expansion of second heart field progenitors, and deletion in cardiomyocytes showed that it was further required for proliferation of ventricular cardiomyocytes in E12.5 hearts (Kwon et al., 2007). β-catenin was also shown to be necessary for the formation of the

atrioventricular canal (AVC) myocardium, and loss-of-function mutants displayed characteristic tricuspid atresia and hypoplastic right ventricle (Gillers et al., 2015).

Thus, the Wnt pathway is a master regulator of the proliferation of both undifferentiated progenitors and embryonic cardiomyocytes. However, Wnt signaling is also key to progenitor cell commitment. In studies of the differentiation of hESCs to cardiomyocytes, it was demonstrated that canonical Wnt signaling (β-catenin-mediated) was required for mesodermal specification, whereas during later differentiation stages it was replaced by non-canonical Wnt signaling (mediated by Ca^{2+}) (Mazzotta et al., 2016). In the absence of exogenous Wnt ligands, β-catenin degradation was shown to be promoted by Glycogen synthase kinase 3 β (Gsk3β), thereby inhibiting activation of Wnt signaling (Wiese et al., 2018). Deletion of Gsk3β (thus activating Wnt signaling) in mice led to a failure of terminal differentiation of cardiomyocytes and hypertrophic cardiomyopathy, suggesting potential teratogenic effects of lithium, which targets GSK3 (Kerkela et al., 2008). Collectively, these studies reveal the importance of temporal and stage-specific Wnt signaling to cardiogenesis. Interestingly, these developmental studies were used as the basis for differentiation protocols of pluripotent stem cells to the cardiomyocyte fate by modulating Wnt signaling (Lian et al., 2012).

2.2.2 TGFβ superfamily

The transforming growth factor β (TGFβ) superfamily consists of secreted factors with known roles in the formation and function of diverse tissues (Hanna & Frangoglannis, 2019). Several exemplary loss-of-function studies of secreted TGFβ family members in the mouse have indicated their role in early cardiogenesis. These include Bone morphogenetic proteins (BMPs), which were initially identified as modulators of bone growth and differentiation (Wozney et al., 1988). Germline deletion of Bmp4 led to a total absence of mesoderm and embryonic lethality prior to E9.5, indicating its necessity in mesodermal specification (Winnier et al., 1995). Similarly, germline deletion of Bmp2 led to embryonic lethality and defects in cardiac formation, indicating non-redundant roles for multiple secreted BMP factors (Zhang & Bradley, 1996). Deletion of the BmpR1a receptor in early mesodermal progenitors resulted in a failure to form the cardiac crescent and first heart field-derived tissues, including the primitive ventricle (Klaus et al., 2007). Thus, TGFβ signaling is integral to early specification of the cardiac lineage.

Further studies have indicated the role of TGFβ signaling in both the definition and growth of cardiac chambers. In zebrafish, deletion of the BMP receptor alk8 resulted in decreased atrial size with no effect on ventricular size, suggesting that BMP signaling also plays a role in antero-posterior morphogenesis (Marques & Yelon; 2009). Germline deletion of Tgfβ2 led to malformation of the heart and death around birth, with anomalies including doublet outlet right ventricle and malformation of the atrioventricular canal (Bartram et al., 2001). When overexpressed in the Nkx2-5+ lineage, Bmp2 promoted cardiomyocyte proliferation and inhibited cardiomyocyte maturation, resulting in E15.5 lethality (Prados et al., 2018). Similarly, germline deletion of Bmp10 resulted in defects in cardiomyocyte proliferation and growth of the ventricular myocardium, causing embryonic lethality (Chen et al., 2004). Collectively, these studies demonstrated distinct roles for BMP family members during both early formation of the heart and proliferation of cardiomyocytes. Similar to Wnt signaling, developmental studies of TGFβ signaling aided the development of protocols for Bmp4/Activin A-driven differentiation of pluripotent stem cells to cardiomyocytes (Kattman et al., 2011).

2.2.3 Retinoic acid/Vitamin A signaling as a negative regulator of progenitor cells and master regulator of anterior-posterior patterning

Retinoic acid is a lipophilic, small molecule derivative of vitamin A which exerts its effects by binding to retinoic acid receptor-retinoid x receptor (RAR-RXR) heterodimers, and these heterodimers themselves act as transcription factors (Niederreither & Dollé, 2008). Interestingly, retinoic acid signaling was the first pathway implicated in heart development, as maternal vitamin A deficiency was shown to result in cardiac malformations in rats (Wilson & Warkany, 1949; Wilson et al., 1953). Excessive maternal vitamin A also generated cardiac malformations in rodents, foreshadowing that the balance of retinoid levels was key to proper formation of the heart (Cohlan, 1953). Indeed, several decades later excessive vitamin A/retinoid intake was reported to underlie congenital heart defects in humans (Lammer et al., 1985; Rothman et al., 1995).

Spatial and temporal localization of retinoic acid signaling during embryogenesis is mediated by enzymes controlling the metabolism of maternal vitamin A into retinoic acid (retinaldehyde dehydrogenases, RALDH1-RALDH3), in addition to the enzymes which degrade retinoic acid (Cytochrome P450 26A1-C1, CYP26A1-C1) (Niederreither & Dollé, 2008). Germline deletion of the retinoic acid synthesis gene Aldehyde dehydrogenase family 1, subfamily A2 (Raldh2) resulted in defects in heart looping and formation of the atrium and sinus venosus, causing embryonic lethality E10.5 (Niederreither et al., 1999; Niederreither et al., 2001). Deletion of both Cyp26a1 and Cyp26c1 in zebrafish resulted in increased numbers of atrial cells in the developing heart, consistent with the phenotype of excess vitamin A. (Rydeen & Waxman, 2014). These mutants also exhibited outflow tract defects and perturbed extracellular matrix in the ventricle (Rydeen & Waxman, 2016). Thus, unlike Wnts and Bmps, retinoic acid signaling during embryogenesis is controlled by the metabolism and degradation of a maternal nutrient.

The presence or absence of retinoic acid signaling determines the total number of CPs and whether CPs assume an atrial or ventricular fate. In mouse and chick embryos, gene expression patterns and retinoid treatments indicated that the retinoic acid synthesis gene Raldh2 determined whether CPs assumed an anterior or posterior fate (Hochgreb et al., 2003). Using the highly tractable zebrafish model, chemical inhibition of retinoic acid signaling increased the number of CPs in the cardiac crescent (Keegan et al., 2005). This was confirmed in Raldh2 homozygous-null mouse embryos, as mutant embryos exhibited defects in atrial development and an expansion of second heart field CPs (Ryckebusch et al., 2008). Early cardiac defects in these mutants could be partially rescued by exogenous retinoic acid treatment, though cardiomyocyte differentiation was still perturbed, purportedly due to alterations in fibroblast growth factor (FGF) signaling (Lin et al., 2010). Retinoic acid has also been shown to be involved in anterior-posterior patterning of the limb bud, suggesting a general role of retinoids in anterior-posterior patterning of developmental structures (Niederreither et al., 2002). The seemingly general role of retinoids in anterior-posterior patterning can be explained by their activation of homeobox (HOX) genes. Importantly, HOX genes were activated by retinoic acid signaling in second heart field progenitors which contribute to distinct anatomical regions of the heart (Bertrand et al., 2011). In zebrafish, hox5b was identified as a specific target of retinoic acid signaling, and the phenotypes of zebrafish embryos with overexpression of hoxb5b are similar to those with excess retinoid exposure (Waxman et al., 2008; Waxman & Yelon, 2009).

Tissue-specific expression of retinoic acid receptors (RARs) and retinoid x receptors (RXRs) also affects the spatial and temporal delineation of retinoic acid signaling, and genetic deletion of these factors has given insight into their role in cardiogenesis (Niederreither & Dollé, 2008). Genetic

deletion of the Rxrα gene resulted in mice with ventricular hypoplasia, ventricular dysfunction, and embryonic death at day 15 of development, in line with the phenotype of maternal vitamin A deficiency (Kastner et al., 1994; Sucov et al., 1994). In a follow-up study, it was observed that these embryos had continued ventricular expression of the atrial isoform of myosin light chain 2, suggesting retinoic acid signaling was involved in governing atrial and ventricular gene expression patterns (Dyson et al., 1995). When a large number of embryos from the same Rxrα knockout mice were studied, a vast array of congenital heart malformations were observed, representing phenocopies of many congenital heart diseases and suggesting that retinoid acid receptor deletions can generate stochasticity (Gruber et al., 1996). Interestingly, when the Rxrα gene was only deleted in cardiomyocytes of the ventricular lineage using Myosin light chain 2 (Myl2)-Cre, there was no phenotype, implying that effects arising from Rxrα deletion either precede Myl2 expression or are due to a lack of the receptor in other cell types in the heart (Chen et al., 1998). Though individual knockout mice for Rarα1 and Rarβ displayed no developmental defects, combinatorial deletion of both Rarα1 and Rarβ resulted in CHD phenotypes consistent with Vitamin A deficiency (Lee et al., 1997). Paradoxically, deletion of retinoic acid receptors in zebrafish subsequently resulted in an increase in retinoic acid signaling and atrialization of the heart, pointing to the complicated feedback mechanisms involved in retinoid signaling (D'Aniello et al., 2013). These studies demonstrate the essential and varied functions of retinoic acid signaling during cardiogenesis.

2.2.4 Other signaling pathways

In addition to the major roles played by Wnt, Bmp, and retinoic acid signaling in cardiogenesis, several additional signaling pathways are necessary for proper heart formation. These include pathways which regulate the proliferation of differentiated cardiomyocytes and thus might be targets for the induction of cardiac regeneration, such as the Notch, Hippo, and insulin signaling pathways. As for previously described signaling pathways, genetic perturbation has given key insights into their role in cardiogenesis and cardiac function. For instance, germline deletion of Notch1 led to defects in ventricular proliferation, whereas overexpression of Notch1 impeded cardiomyocyte differentiation and maturation (Grego-Bessa et al., 2007). Specific deletion of Notch signaling in cells of the neural crest lineage led to defects in the formation of smooth muscle cells, in addition to causing congenital heart defects of the aortic arches, pulmonary arteries, and ventricular septum (High et al., 2007). Interestingly, the activation of Notch signaling in chamber cardiomyocytes resulted in activation of genes of the conduction system as well as reprogramming of the electrophysiological phenotype (Rentschler et al., 2012). FGF signaling also regulated cardiomyocyte subtype fate decisions, as it was shown to be integral to the maintenance of the ventricular fate via activation of Nkx transcription factors, which suppress atrial genes (Pradhan et al., 2017).

Similar to Notch signaling, Hippo and insulin pathways have also been implicated in embryonic cardiomyocyte proliferation. Embryonic deletion of the Hippo effector Yap1 resulted in decreased cardiomyocyte proliferation and myocardial hypoplasia, whereas Yap1 overexpression induced cardiomyocyte proliferation (von Gise et al., 2012). Similarly, Insulin growth factor (IGF) secreted by the epicardium was shown to direct the proliferation of ventricular cardiomyocytes from E11.5-E14.5 of development (Li et al., 2011). Some signaling pathways typically associated with their role in adult tissue function also exhibit roles in cardiogenesis, such as endothelin-1 (ET-1). ET-1 is a 21-amino acid, secreted, vasoconstrictive peptide initially isolated from aortic endothelial cells which is activated during pathological processes in the heart (Kaye & Krum, 2007; Yanagisawa et al., 1988). Interestingly, ET-1 also has a developmental role, and mice lacking ET-1 develop outflow tract and ventricular-septal defects, in addition to defective sympathetic innervation of the heart (Ieda et al.,

2004; Kurihara et al., 1995). Collectively, these studies demonstrate that diverse signaling pathways are involved in cardiomyocyte differentiation, proliferation, and function.

2.2.5 Examples of cross-talk between developmental signaling pathways during cardiogenesis

Signaling pathways in the heart do not act independently, and there are myriad examples of positive or negative regulation of one signaling pathway over another during cardiogenesis. For instance, BMP signaling was shown to activate Wnt signaling during cardiomyocyte differentiation, as Activin A/BMP4 treatment of human PSCs resulted in the upregulation of canonical Wnt signaling (Paige et al., 2010). Similarly, retinoic acid receptor depletion led to upregulation of TGFβ signaling, thereby affecting both apoptosis and renewal of cardiac progenitors (Kubalak et al., 2002; Li et al., 2010). Furthermore, Wnt signaling in cardiac progenitors was shown to be inhibited by Notch signaling, resulting in promotion of differentiation and suppression of progenitor self-renewal (Kwon et al., 2009; Kwon et al., 2011). Similarly, Hippo signaling inhibited cardiomyocyte proliferation and controlled heart size by negative repression of Wnt signaling (Heallen et al., 2011). Due to the seeming overlap of all signaling pathways, cross-regulatory signaling circuits are difficult to study systematically, but improved single-cell spatiotemporal detection methods might hold the key to understanding how signaling pathway thresholds and regulatory balance govern phenotype during embryogenesis and in the diseased heart.

2.2.6 Developmental signaling pathways in adult heart disease

The signaling pathways underlying formation of the heart also play key roles in compensatory disease processes which occur during heart failure. Functional genetics in mouse models has led to an improved understanding of the role of signaling pathways in the adult heart, and the relationship between embryonic and adult phenotypes is summarized in Table 3. For instance, inactivation of the Wnt effector β-catenin in cardiomyocytes following myocardial infarction led to improved cardiac function (Zelarayán et al., 2008). Deletion of Gsk3β, which leads to the activation of Wnt signaling, was reported to induce cardiomyocyte proliferation but also cardiac hypertrophy (Kerkela et al., 2008). Additionally, overexpression of Gsk3β (thus inactivating Wnt signaling), was reported to prevent pathological cardiac hypertrophy (Antos et al., 2002). These results suggest that negative effects arise due to re-activation of developmental Wnt signaling in the failing heart.

The role of Hippo signaling in regulating cardiomyocyte proliferation in the embryonic heart has led to intensive investigation of its role in cardiac regeneration. Deletion of Yap, a transcriptional effector of the Hippo pathway, impeded neonatal mouse cardiac regeneration, and overexpression of Yap promoted regeneration (Xin et al., 2013). Moreover, iterative deletion of other Hippo signaling components in the postnatal heart led to improved regenerative capacity (Heallen et al., 2013). In a separate study, loss of Salv improved heart function following myocardial infarction in the mouse (Leach et al., 2017). Strikingly, overexpression of an active form of Yap (Yap5sa) in the adult heart led to a re-entry of adult cardiomyocytes into the cell cycle, resulting in myocardial hyperplasia and thickening of the ventricular wall (Monroe et al., 2019). However, mice died four days after initiation of expression due to heart failure, shedding doubt on the benefits of inducing unrestrained cardiomyocyte proliferation in the adult mammalian heart (Monroe et al., 2019). Similarly, mice lacking components of the Hippo signaling pathway were also shown to exhibit cardiac de-differentiation when subjected to pressure overload, exacerbating the disease phenotype (Ikeda et al., 2019). Interestingly, evolutionary pressures related to endothermy appear to regulate the regenerative capacity of the heart. Recent studies have indicated that the lack of regenerative capacity of the mammalian heart is related to increased thyroid signaling during the acquisition of endothermy

(Hirose et al., 2019). In mice, thyroid signaling also regulated cardiomyocyte proliferation and binucleation, and mice lacking thyroid hormone have improved cardiomyocyte proliferation in neonatal and adult hearts, in addition to improved function after myocardial infarction (Hirose et al., 2019). Collectively, these studies have demonstrated that modulation of developmental signaling pathways in the adult heart can be used to stimulate developmental processes, like proliferation, though the benefits of this approach are controversial.

ET-1, a developmentally expressed neurohormone, is integral to adult cardiac function and has been investigated extensively due to its activation in the failing heart (Kaye & Krum, 2007). ET-1 displayed a positive inotropic effect on the atria of guinea pigs, and the receptor was expressed in neonatal rat cardiomyocytes (Ishikawa et al., 1988; Hirata et al., 1989). Endogenous ET-1 also regulated increases in contractility due to increased coronary flow in perfused hearts, though differing effects occurred if acting on ETA or ETB receptors (Piuhola et al., 2003). In addition to contractility, ET-1 regulated force-generation in normal and hypertrophic hearts (Piuhola et al., 2003). Furthermore, ET-1 increased the expression of atrial natriuretic peptide (ANP) and sarcomeric genes in rat cardiomyocytes, in addition to stimulating cellular hypertrophy (Fukuda et al., 1989; Ito et al., 1991). Finally, ET-1 was shown to regulate Ca^{2+} oscillations and heart rate in embryonic cardiomyocytes (Karppinen et al., 2014). Thus, in addition to regulating morphogenesis, endothelin signaling is a major regulator of cardiovascular physiology in adults.

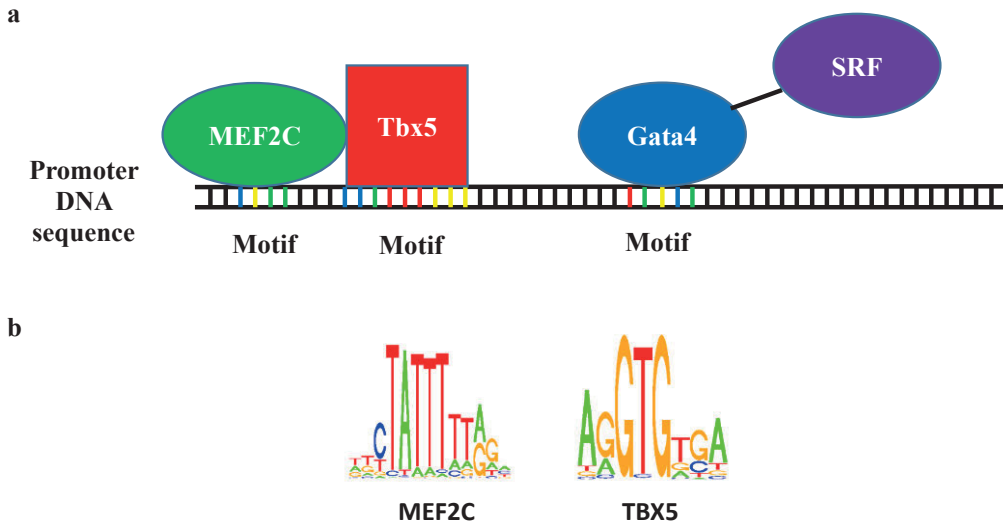

Figure 4. Function of transcription factors. TF:TF interactions can result in synergistic activation or antagonistic repression. This can occur as a result of DNA binding of both TFs to nearby segments of DNA, or binding of only a single factor. **b** TFs bind to unique DNA sequences called DNA motifs. Analysis of these DNA sequences can be used to identify potential binding sites of TFs. Myocyte enhancer factor 2c (Mef2c), T-box transcription factor 5 (Tbx5), GATA transcription factor 4 (Gata4), Serum response factor (Srf).

gene	Role in multipotent cardiac progenitors/early cardiogenesis	Role in differentiated cardiomyocytes/late cardiogenesis	Role in postnatal heart	Human disease associations	citations
Gata4	Germline deletion leads to altered formation of linear heart tube and lethality E7-E9.5.	Deletion in Nkx2-5+ or Mhc+ lineages leads to impeded cardiomyocyte proliferation and myocardial thinning. Results in heart failure postnatally.	Overexpression results in cardiomyocyte hypertrophy. Postnatal deletion leads to cardiomyocyte hypoplasia and pressure-overload induced apoptosis.	ventricular-septal defects, atrioventricular septal defects, valve defects	Bisping et al., 2006; Garg et al., 2003; Liang et al., 2001; Molkentin et al., 1997; Oka et al., 2006; Zeisberg et al., 2005
Hand1	Germline deletion results in impaired development of the left ventricle.	Deletion in Nkx2-5 + or Mhc+ lineages results in impaired formation of cardiac conduction system and interventricular septum.	Germline deletion results in postnatal death due to cardiac dysfunction.	-	Firulli et al., 2019; McFadden et al., 2005
Hand2	Germline deletion leads to defects in right ventricular formation, lethality E10.5.	-	-	-	Srivastava et al., 1997; Yamagishi et al., 2000
Hey2	Ventricular-specific gene involved in the repression of atrial gene program.	Ventricular-specific gene involved in the repression of atrial gene program, germline deletion results in impeded proliferation and thinning of the myocardial wall.	Overexpression attentuates hypertrophic response to phenylephrine. Inhibits Gata4.	-	Koibuchi & Chin, 2007; Xiang et al., 2006
Irx4	Ventricular-specific gene involved in the repression of atrial gene program. Expression begins E7.5.	Ventricular-specific gene involved in the repression of atrial gene program.	Germline deletion results in postnatal cardiomyopathy.	-	Bao et al., 1999; Bruneau et al., 2000; Bruneau et al., 2001
Mef2c	Germline deletion leads to defective formation of the right ventricle and looping defects.	No phenotypic consequences when deleted.	Overexpression leads to dilated cardiomyopathy or increased sensitivity to pressure overload. Activity decreased in border zone following myocardial infarction.	-	van Duijvenboden et al., 2019; Xu et al., 2006
Nkx2-5	Germline deletion leads to defects in looping and onset of ventricular gene expression.	Myocardial deletion results in impaired development of the cardiac conduction system.	-	atrial-septal defects, ventricular-septal defects, tetralogy of fallot	Jay et al., 2004; Lyons et al., 1995; Pashmforoush et al., 2004; Schott et al., 1998

Table 4 (Continued on next page). Cardiovascular effects of deletion of transcription factors during early/late embryogenesis and adulthood in mouse studies. Additionally, roles of these transcription factors in human congenital heart diseases. These studies laid the framework for the identification of the core cardiac gene regulatory network. Additionally, they showed the distinct role of transcription factors in the development of subregions of the heart. GATA transcription factor 4 (Gata4), Heart and neural crest derivatives expressed 1 (Hand1), Heart and neural crest derivatives expressed 2 (Hand2), hes related family basic helix-loop-helix transcription factor with YPRW motif 2 (Hey2), Iroquois homeobox 4 (Irx4), Myocyte enhancer factor 2 (Mef2c), NK2 homeobox 5 (Nkx2-5), Nuclear receptor subfamily 2 group F member 2 (Nr2f2), Paired like homeodomain 2 (Pitx2), Serum response factor (Srf), T-box transcription factor 5 (Tbx5).

gene	Role in multipotent cardiac progenitors/early cardiogenesis	Role in differentiated cardiomyocytes/late cardiogenesis	Role in postnatal heart	Human disease associations	citations
Nr2f2	Atrial-specific expression, master regulator of atrial fate, germline deletion results in reduced size of atria, embryonic lethality.	Cardiomyocyte-specific deletion leads to upregulation of ventricular gene expression in the atria, ventricular-like electrophysiology in the atria.	-	-	Pereira et al., 1999; Wu et al., 2013
Pitx2	Positioning of the heart, looping, and left-right asymmetry.	-	Deletion in neonatal heart impedes regeneration.	-	Ai et al., 2006; Galli et al., 2008; Lin et al., 1999; Logan et al., 1998; Kitamura et al., 1999; Tao et al., 2016
Srf	Germline deletion leads to failure to form mesoderm.	Cardiomyocyte-specific deletion leads to defeciencies in sarcomeric gene expression, sarcomere assembly, and increased embryonic lethality.	Cardiomyocyte-specific deletion leads to reduced contractility, cardiomyopathy, and fatal heart failure. Cardiomyocyte-specific overexpression leads to hypertrophic cardiomyopathy.	-	Arsenian et al., 1998; Niu et al., 2005; Parlakian et al., 2005; Zhang et al., 2001
Tbx5	Germline deletion leads to malformation of atria and left ventricle, Embryonic lethality.	Ventricle-specific deletion leads to defects in left-right patterning. Tbx5 haploinsufficiency underlies conduction system defects, atrial-septal defects, ventricular-septal defects, limb defects.	Deletion of Tbx5 in adult mice leads to sustained atrial fibrillation. Deletion from ventricular conduction system leads to arrythmias. Regulates diastolic function.	Holt-Oram syndrome	Arnolds et al., 2012; Bruneau et al., 1999; Bruneau et al., 2001; Takeuchi et al., 2003; Moskowitz et al., 2004; Koshiba-Takeuchi et al., 2009; Nadadur et al., 2016; Xie et al., 2012; Zhu et al., 2008

2.3 Tissue-specific transcription factors composing the cardiac gene regulatory network: regulators of congenital and adult heart diseases

Developmental signaling pathways activate downstream gene regulatory networks composed of tissue-specific, cardiac developmental transcription factors (TFs). These TFs bind to promoters and enhancers of cell identity genes at characteristic motifs, and may act synergistically to promote specific phenotypic effects (Figure 4a-b). Central to the understanding of heart development and malformation are the expression patterns, loss- and gain-of-function phenotypes, and congenital heart disease manifestations of these TFs. As many of these networks are re-activated in the context of heart failure, knowledge from developmental studies can provide insight into the molecular underpinnings of adult disease. Notably, loss-of-function mouse studies combined with analysis of embryological cardiac defects has led to a detailed mapping of the core cardiac gene regulatory

network. Additionally, the role of many of these TFs in cardiac regeneration and adult heart disease has been explored. A summary of mouse studies with tissue-specific gain- and loss-of-function phenotypes for various TFs is shown in Table 4. Though not comprehensive, this section describes the major TFs comprising the core cardiac TF network. In contrast to developmental signaling pathways, which are often ubiquitous across tissue types, the TFs described here are often tissue-specific and serve as master regulators of the cardiac phenotype. It is this specificity that holds great promise for their use in developing targeted therapies.

2.3.1 Mesodermal transcription factors preceding the onset of expression of the core cardiac network

As cardiac tissue arises from the mesodermal germ layer, analysis of the TFs comprising this tissue is a logical point of departure. Homozygous mutants for the T/Brachyury gene have been known since the 1930s to lead to defects in mesodermal (notochord) formation and embryonic lethality (Chesley, 1935). This gene was later identified as a nuclear protein specifically expressed in the gastrulating embryo but subsequently downregulated during later development (Kispert & Herrmann, 1994; Wilkinson et al., 1990). Importantly, T/Brachyury was identified as a target of Wnt/β-catenin signaling in differentiating mESCs, serving as a linkage between developmental signals and the activation of downstream mesodermal transcription factors (Arnold et al., 2000).

The earliest cardiac-restricted transcription factor is MESP1. Mesp1 was initially identified as a mesodermal-specific gene expressed during E6.5-E7.5 of mouse embryogenesis, and was subsequently demonstrated to be the earliest marker of cardiac mesoderm (Bondue et al., 2008; Saga et al., 1996). In mutants lacking Mesp1, cardiac mesodermal cells failed to migrate and form the linear heart tube (Saga et al., 1999). Similarly, overexpression of Mesp1 in mESCs led to increased differentiation to the cardiomyocyte fate via repression of Wnt signaling and activation of core cardiac TFs (David et al., 2008). Combined deletion of both Mesp1 and Mesp2 resulted in the complete absence of cardiac mesoderm and embryonic lethality (Kitajima et al., 2000). Importantly, Mesp1 was shown to promote cardiovascular, but not hematopoietic fates within mesodermal populations (Lindsley et al., 2008). Collectively, these studies show that Mesp1 is the earliest marker of general cardiac mesoderm, and that it is also a key TF regulating specification of the cardiovascular lineage.

A remarkable achievement in early embryological studies was the discovery that different regions of the heart arise from different mesodermal populations expressing or lacking Isl1. Similar to Brachyury, Isl1 was shown to be activated by canonical Wnt signaling, serving as an additional bridge between morphogens and TF activation (Lin et al., 2007). In a landmark study, it was observed that mice lacking Isl1 fail to develop the outflow tract, right ventricle, and parts of the atria (Cai et al., 2003). In the same study, lineage tracing revealed that Isl1+ cells gave rise to these specific regions of the heart, but not to the left ventricle (Cai et al., 2003). This study revealed that divergent progenitor populations in early embryos give rise to distinct anatomical structures. Subsequently, it was discovered that Isl1+ cells arising from the secondary heart field could be clonally expanded and differentiated into cardiomyocyte, smooth muscle, and endothelial cell lineages (Moretti et al., 2006). Despite the very intuitive first/second heart field dichotomy, it was later shown that Isl1+ cells also included cells of neural crest cell origin (Engleka et al., 2012).

2.3.2 TBX5 – Master regulator of left ventricular morphogenesis, septation, and cause of Heart-Hand syndrome

Both mouse and human studies have revealed that TBX5 is an integral member of the core cardiac TF network. Human genetics studies revealed that loss-of-function mutations in TBX5 caused Holt-Oram syndrome, a developmental disorder characterized by heart and upperlimb malformation (Basson et al., 1997; Li et al., 1997). Interestingly, heterozygous Tbx5 deletion resulted in forelimb and congenital heart malformations which were a phenocopy of Holt-Oram syndrome (Bruneau et al., 2001). Additionally, Tbx5 expression during mouse embryogenesis revealed preferential expression in the developing atria, left ventricle, and interventricular septum, and germline homozygous deletion of Tbx5 resulted in embryonic lethality due to impaired growth of these structures (Bruneau et al., 1999; Bruneau et al., 2001). Overexpression studies demonstrated that the absence of Tbx5 in the right ventricle is necessary for its proper formation, in addition to showing that left-ventricle-restricted Tbx5 expression is important for correct formation of the ventricular septum (Takeuchi et al., 2003). Similarly, ventricle-specific deletion of Tbx5 led to a mis-patterned ventricle without characteristic morphological differences between the right and left chambers (Koshiba-Takeuchi et al., 2009). In addition to chamber formation, Tbx5 was also necessary for formation of the conduction system and the septa. Tbx5 haploinsufficiency caused failed maturation of the atrioventricular node, leading to conduction system defects in mice and humans (Moskowitz et al., 2004). Additionally, Tbx5 haploinsufficiency in the second heart field, but not the first heart field, resulted in atrial-septal defects (Xie et al., 2012). Collectively, these studies show the chamber-specific role of Tbx5 in embryonic heart formation.

Other T-box factors also play important roles in heart development and cardiomyocyte subtype-specific gene expression, and there is evidence for the relationship between T-box factors and retinoic acid signaling. Loss of Tbx1 was shown to underlie the phenotype of DiGeorge syndrome, a disease characterized by heart and craniofacial defects (Merscher et al., 2001). Interestingly, Tbx5 was shown to be activated by Tbx1 and retinoic acid signaling, and inhibition of retinoic acid signaling resulted in the absence of Tbx5 and the development of atrial-septal defects (De Bono et al., 2018). Ripply3, which in zebrafish is a recently identified repressor of T-box TFs, was shown to be a target of retinoic acid signaling and to repress the differentiation of ventricular cardiomyocytes (Song et al., 2019). Tbx2 and Tbx3 have also been reported to control the differentiation of the conduction system. Tbx3 was shown to be specifically expressed in the cardiac conduction system, and deletion of Tbx3 resulted in aberrant conduction system development (Bakker et al., 2008; Hoogaars et al., 2004). Furthermore, Tbx3 repressed the atrial cardiomyocyte gene expression program and promoted sinoatrial node gene expression and physiology (Hoogaars et al., 2007). Strikingly, transgenic overexpression of Tbx3 reprogrammed working myocardium into pacemaker cells (Bakker et al., 2012). In addition to Tbx3, Tbx2 is also important to conduction system development. Cre-based lineage tracing demonstrated that Tbx2+ cells of the atrioventricular canal gave rise to the atrioventricular node (Aanhaanen et al., 2009). Deletion of Tbx2 resulted in upregulation of working myocardium genes in the atrioventricular canal, leading to the formation of accessory conduction pathways and premature ventricular excitation (Aanhaanen et al., 2011). Finally, Tbx18 was shown to only be expressed in tissues lacking Nkx2-5 and to be necessary for the formation of the sinus horns in the venous pole of the heart (Christoffels et al., 2006). Thus, T-box factors represent an important family of TFs with chamber-specific and cell-type specific functions.

2.3.3 MEF2 factors

MEF2 factors were among the first TFs shown to be expressed in the cardiac mesoderm during early embryogenesis, as well as in the fully formed heart (Edmondson et al., 1994). The presence of MEF2 binding sites within cardiac promoters indicated their role as cardiac TFs, and this was confirmed in cultured neonatal cardiomyocytes and *in vivo* (Molkentin & Markham, 1993). Expression was also observed in cultured rat aortic smooth muscle cells, and MEF2B was shown to regulate smooth muscle gene expression (Firulli et al., 1996; Katoh et al., 1998). Detailed genetic studies have revealed an extensive role for MEF2 factors in the cardiovascular system, including that of Mef2c. Homozygous deletion of Mef2c in mouse embryos resulted in non-formation of the right ventricle, a failure of rightward looping of the heart tube, and downregulation of the cardiomyocyte gene expression program (Lin et al., 1997). An enhancer of Mef2c was used for Cre-mediated lineage tracing, labelling a subset of cells of the secondary heart field (anterior heart field) which gave rise to the right ventricle, outflow tract, and ventricular septum (Verzi et al., 2005). Interestingly, in this anterior heart field subdomain Mef2c is upregulated by a combination of Isl1 and Gata transcription factors (Dodou et al., 2004), indicating that it is a marker of late progenitor cells preceding the formation of differentiated myocardium. Importantly, these studies showed that MEF2C is a master regulator of right, rather than left ventricular fate, in contrast to TBX5. In addition to the formation of cardiac muscle, MEF2C is also important to vessel formation. Mef2c (-/-) mutant mice failed to develop vasculature, and conditional deletion of Mef2c in endothelial cells resulted in inhibition of transfenestral migration of smooth muscle cells in carotid arteries (Lin et al., 1997; Lu et al., 2017). Interestingly, smooth muscle cell differentiation was also completely impeded (Lin et al., 1997). Thus, MEF2C is integral to the differentiation of diverse cardiac cell types and is critical to formation of the right ventricle. In addition to its cardiac function, Mef2c is expressed in terminally differentiated neurons, and conditional deletion in mice resulted in behavioral defects (Harrington et al., 2016; Lyons et al., 1995). In a separate study, conditional deletion of Mef2c in the brain resulted in defects in learning and memory, in addition to an increase in the number of excitatory synapses (Barbosa et al., 2008). The role of other MEF2 transcription factors in cardiogenesis has also been explored. For instance, most embryos with a homozygous deletion in Mef2a died because of right ventricular defects, while those reaching adulthood suffered from mitochondrial defects and arrhythmias (Naya et al., 2002). Thus, MEF2 factors are integral to cardiogenesis, and MEF2C is a specific mediator of right ventricular development and gene regulation.

2.3.4 GATA4

GATA4 is a zinc finger TF and member of the core cardiac TF network initially identified by its upregulation in E6.5 embryos *in vivo*, in addition to its upregulation following retinoic acid stimulation and differentiation of PSCs into embryoid bodies (Arceci et al., 1993). GATA4 was subsequently shown to upregulate α-myosin heavy chain (α-MHC) expression in cultured neonatal cardiomyocytes, and DNA binding of GATA4 was itself increased by both exogenous retinoic acid and triiodothyronine stimulation (Molkentin et al., 1994). Germline deletion of Gata4 in mice resulted in perturbed formation of the linear heart tube and lethality between E7-E9.5, whereas specific deletion of Gata4 in Nkx2-5-Cre or MHC-Cre lineages resulted in right ventricular myocardial thinning due to decreased cardiomyocyte proliferation (Molkentin et al., 1997; Zeisberg et al., 2005). Thus, GATA4 is a regulator of both cardiomyocyte differentiation and cardiomyocyte proliferation. Interestingly, GATA4 and the related transcription factor GATA6 showed partial redundancy. Compound, but not single heterozygosity at the Gata4 and Gata6 loci resulted in embryonic lethality at E13.5 of development due to cardiovascular defects such as thinned myocardium and malformation

of the septa (Xin et al., 2006). Strikingly, deletion of both Gata4 and Gata6 completely impeded heart formation, resulting in acardia (Zhao et al., 2008). In addition to these roles, Gata4 was also shown to be a primary regulator of Hedgehog signaling in the second heart field and outflow tract (Liu et al., 2019; Zhou et al., 2017).

2.3.5 NKX2-5

Nkx2-5 was identified as a marker of the earliest myocardial progenitor cells located in the cardiac crescent of E7.5 embryos, and Nkx2-5 expression was itself shown to be dependent on expression of GATA4 (Lien et al., 1999; Lints et al., 1993; Searcy et al., 1998). Because of its early expression throughout the cardiac lineage, various mouse and human PSC lines have used Nkx2-5 to track cardiomyocyte differentiation (Elliot et al., 2011; Hidaka et al., 2003; Hsiao et al., 2008). Additionally, genetic studies of Nkx2-5 have provided insight into its function. Homozygous deletion of Nkx2-5 resulted in embryos with cardiac looping defects and downregulation of the ventricle-specific gene Myl2 (Lyons et al., 1995; Tanaka et al., 1999). Importantly, Nkx2-5 was shown to negatively regulate cardiac progenitors via the repression of Bmp2/Smad1 signaling, leading to the onset of the differentiation program (Prall et al., 2007). Intriguingly, Nkx2-5 was itself activated by TGFβ signaling, indicating the existence of a negative feedback loop (Liberatore et al., 2002; Monzen et al., 1999). Additionally, heterozygous Nkx2-5 mutants sometimes displayed congenital heart defects, such as atrial-septal dysmorphogenesis (Biben et al., 2000). Furthermore, human mutations in the Nkx2-5 DNA-binding domain led to congenital heart disease, manifested as atrial-septal defects, ventricular-septal defects, and tetralogy of fallot (Schott et al., 1998). The levels of NKX2-5 also determined the number of conduction system cells in the sinoatrial node, and complete deletion of Nkx2-5 led to an absence of sinoatrial node progenitors (Jay et al., 2004). Similarly, deletion of Nkx2-5 in ventricular myocardium led to atrioventricular block and cardiomyopathy, but did not cause the ventricular- and atrial septal defects characteristic of germline deletion of Nkx2-5, alluding to the importance of progenitor expression of Nkx2-5 to formation of these structures (Pashmforoush et al., 2004). Thus, stage-specific expression of Nkx2-5 is integral to ventricular and conduction system formation, in addition to formation of the septa.

2.3.6 Transcription factors determining left-right asymmetric gene expression programs in the heart

Gene expression programs between the left and right sides of the heart differ during embryogenesis and adulthood, and this specificity is controlled by tissue-specific TFs, such as Heart and neural crest derivatives expressed 1 & 2 (HAND1 and HAND2). Hand1 and Hand2 are expressed in complementary patterns in the embryonic heart, as Hand1 was shown to be expressed in left ventricle-forming tissue and Hand2 in right ventricle-forming tissue (Srivastava et al., 1995; Srivastava et al., 1997). Importantly, deletion of Hand1 resulted in morphological abnormalities of the left ventricle, abnormal ventricular gene expression, and neonatal lethality due to cardiac dysfunction (McFadden et al., 2005). In contrast, deletion of Hand2 resulted in malformation of the right ventricle and vasculature, as well as embryonic lethality at E10.5 of development (Srivastava et al., 1997; Yamagishi et al., 2000).

The Paired like homeodomain 2 (PITX2) transcription factor was also shown to be a key regulator of left-right asymmetry. Pitx2 was shown to be a downstream target of Shh and Nodal signaling, and it was specifically expressed on the left side of the lateral plate mesoderm (Logan et al., 1998). Strikingly, overexpression of Pitx2 in the right side of the chick heart impeded cardiac looping (Logan et al., 1998). Furthermore, deletion of Pitx2 resulted in embryos with positioning of the heart on the

right side of the body, in addition to failed atrial septation and defects in other organs (Lin et al., 1999). Additional mutant models have implicated PITX2 as a master regulator of cardiac asymmetry of ventricles and atria, in addition to the regulation of valve formation and formation of the pulmonary myocardium (Ai et al., 2006; Galli et al., 2008; Kitamura et al., 1999; Mommersteeg et al., 2007). Thus, HAND factors and PITX2 are instrumental to the definition of right/left identity in the heart.

2.3.7 Transcription factors mediating atrial/ventricular-specific gene expression programs

A few TFs have been identified with ventricular-specific gene expression patterns which repress the expression of atrial genes, such as Iroquois homeobox 4 (Irx4) and hes related family basic helix-loop-helix transcription factor with YPRW motif 2 (Hey2). IRX4 is restricted to the ventricles during embryogenesis and was shown to regulate the expression of chamber-specific contractile proteins (Bao et al., 1999). Additionally, deletion of Irx4 resulted in cardiac hypertrophy and impaired contractility in adult mice, in addition to upregulation of the atrial-specific Slow myosin heavy chain 3 (SMyHC3) transgene (Bruneau et al., 2001). Irx4 expression was downregulated in Nkx2-5 (-/-) mouse embryos, in addition to Hand2 (-/-) embryos, indicating that it was downstream of the core cardiac TF network (Bruneau et al., 2000). Similar to Irx4, Hey2 was shown to be restricted to ventricular cardiomyocytes, and when deleted resulted in expression of atrial genes in the ventricles (Koibuchi & Chin, 2007; Xin et al., 2007). When Hey2 was overexpressed in the atria, it resulted in repression of the atrial gene expression program, implicating Hey2 as a repressor of the atrial phenotype (Xin et al., 2007). In contrast to Irx4 and Hey2, Nuclear receptor subfamily 2 group F member 2 (Nr2f2) was specifically expressed in the atria and was shown to be a repressor of ventricular gene expression patterns. Germline deletion of Nr2f2 resulted in a stark reduction in size of the primitive atria, and cardiomyocyte-specific deletion of Nr2f2 during embryogenesis resulted in an upregulation of ventricular cardiomyocyte genes in the atria and ventricular-like physiological characteristics of atrial cells (Pereira et al., 1999; Wu et al., 2013). Thus, atrial and ventricular-specific TFs delimit the expression domains of atrial and ventricular genes.

2.3.8 Additional members of the cardiac transcription factor network

In addition to those mentioned, functional genetics studies have identified many additional transcriptional regulators of cardiogenesis, such as serum response factor (SRF), myocardin, homeodomain-only protein (HOPX), nuclear factor of activated T cells (NFAT), and forkhead factors. Notably, mouse embryos lacking Srf did not express mesodermal markers (Arsenian et al., 1998). Additionally, cardiomyocyte-specific deletion of Srf during embryogenesis led to death at E13.5 due to myocardial thinning, chamber dilation, and abnormally developed trabeculae and interventricular septum (Niu et al., 2005; Parlakian et al., 2004). A coactivator of SRF, myocardin is a transcription factor which was shown to be expressed in cardiac and smooth muscle cells and form a complex with SRF to activate both the smooth muscle Sm22 promoter and cardiac Nppa promoters (Wang et al., 2001). Deletion of the homeobox gene Hop, which is downstream of Nkx2-5, resulted in embryonic lethality E10.5-E12.5 due to pericardial effusion (Chen et al., 2002, Shin et al., 2002). Interestingly, Hopx was shown to be a marker of unipotent cardiomyocyte progenitors (cardiomyoblasts) and to be involved in the suppression of Wnt signaling (Jain et al., 2015). NFAT and forkhead TFs were also shown to be significant to heart development. Deletion of both Nfatc3 and Nfatc4 resulted in E10.5 lethality due to thin ventricles, decreased ventricular cardiomyocyte proliferation, and mitochondrial defects (Bushdid et al., 2003). Finally, cardiomyocyte-specific deletion of the Forkhead box m1 (Foxm1) TF resulted in embryonic lethality between E14.5 and birth due to proliferation defects and

ventricular hypoplasia (Bolte et al., 2011). Though not comprehensive, the large number of TFs described in these studies indicates the overall complexity of cardiac gene regulatory networks.

2.3.9 RNAs as regulators of cardiac gene transcription

Noncoding RNAs have also been identified as regulators of cardiogenesis, including microRNAs and long non-coding RNAs (lncRNAs). For instance, the microRNA miR-1 regulated cardiomyocyte proliferation by modulating the mRNA levels of Hand2, and loss-of-function of miR-1 caused ventricular-septal defects (Zhao et al., 2005). Additionally, deletion of miR-133 led to reduced cardiomyocyte proliferation and caused congenital abnormalities, purportedly by decreasing levels of Srf (Liu et al., 2008). Loss of miR-17 led to a variety of developmental defects, including ventricular-septal defects (Ventura et al., 2008). Finally, the Let-7 family of microRNAs were upregulated following one-year of *in vitro* maturation of stem cell-derived cardiomyocytes, and overexpression of Let-7g promoted cell maturation (Kuppusamy et al., 2015). Thus, several microRNAs have been identified that regulate cardiogenesis and cardiac function.

In addition to microRNAs, some long non-coding RNAs (lncRNAs) have been implicated in cardiac development and postnatal cardiac function. Deletion of the lncRNA Fendrr resulted in defects in body wall closure, but also caused myocardial thinning (Grote et al., 2013). The same study reported altered transcription of cardiac TFs and interactions between Fendrr and epigenetic complexes, suggesting that lncRNAs might function upstream of cardiac TFs (Grote et al., 2013). Another lncRNA, Braveheart, was shown to have a role in the cardiac differentiation of mESCs as well as the maintenance of cardiac gene regulatory networks in neonatal cardiomyocytes (Klattenhoff et al., 2013). Interestingly, many lncRNAs were also described as transcribed enhancers, and many thousands of lncRNAs have been identified whose transcription is dependent upon TBX5 (Yang et al., 2017). When performing genetic deletion of lncRNAs, it is therefore imperative to distinguish between the functional requirements of the enhancer region and the transcribed lncRNA in question, as demonstrated by two recently identified lncRNAs associated with the HAND2 transcription factor (Anderson et al., 2016; Ritter et al., 2019). Collectively, these studies suggest a functional role of lncRNAs in cardiogenesis and cardiovascular function, though the precise mechanisms have not yet been characterized.

2.3.10 Post-translational modifications of core cardiac transcription factors

Post-translational modifications of cardiac TFs affect not only protein levels, but also function. Evidence for this is provided by studies of histone deacetylases (HDACs), which remove acetyl moieties from both histones and TFs. Treatment of mESCs with trichostatin A, an HDAC inhibitor, resulted in increased acetylation of the cardiac TF GATA4 and increased expression of both an Nkx2-5 reporter and sarcomeric genes (Kawamura et al., 2005). Furthermore, hyperacetylation of GATA4 resulted in pathological cardiomyocyte proliferation and lethality, and this was prevented by HOPX and HDAC2 (Trivedi et al., 2010). Zebrafish studies showed that lysine acetyltransferase 2 A (KAT2A) and lysine acetyltransferse 2 B (KAT2B) acetylated TBX5 at Lys339, increasing its transcriptional activity (Ghosh et al., 2018). Interestingly, HDAC4 and HDAC5 deactetylated TBX5, subsequently decreasing its transcriptional activity (Ghosh et al., 2019).

Phosphorylation induced by intracellular kinases also affects the activity of cardiac transcription factors. MEF2 factors are key mediators of the hypertrophic response in cardiomyocytes, and MEF2 activity is enhanced as a result of post-translational modifications induced by mitogen activated protein kinases (MAPKs) (Lu et al., 2000). MEF2C was shown to be phosphorylated at Ser-59, and

this modification increased both its DNA binding and transcriptional activity (Molkentin et al., 1996). Phosphorylation of serine-105 by MAPK signalling resulted in increased GATA4 binding and transcription, causing cardiomyocyte hypertrophy in certain contexts (Liang et al., 2001). Interestingly, post-translational modifications of TFs also affect protein:protein interactions. For instance, HDAC4 inhibited the interaction of TBX5 with MEF2C (Ghosh et al., 2019). Thus, posttranslational modifications have been shown to serve as an important mechanism for the regulation of transcriptional activity of core cardiac TFs.

2.3.11 Transcriptional synergy of core cardiac transcription factors

Transcription factors in the heart possess both synergistic and antagonistic functions that have been characterized as physical protein:protein interactions at the promoters of target genes. In addition to providing insight into the onset of both congenital and adult heart disease, these interactions also illustrate how eukaryotic transcriptional networks function. In a landmark study, GATA4 and NKX2-5 physically interacted and synergistically activated the Nppa promoter, an important biomarker of heart failure (Lee et al., 1998). Other examples of TF:TF interactions shown to occur at the Nppa promoter include TBX5:NKX2-5, GATA4:HAND2, and MEF2C:HAND2 (Dai et al., 2002; Hiroi et al., 2001; Zang et al., 2004). More than two TFs have also been shown to interact in concert, as SRF was shown to activate cardiac gene expression in cooperation with GATA4 and NKX2-5 (Sepulveda et al., 2002). TBX5 and GATA4 were also shown to physically interact, and mutations in Gata4 which prevented the recruitment of TBX5 to cardiac super-enhancers resulted in initation of endothelial gene expression networks at the expense of cardiomyocyte gene activation (Ang et al., 2016). TBX5 and MEF2C also physically interacted, and this interaction was critical for sarcomeric gene activation and cardiomyocyte differentiation (Ghosh et al., 2009).

Embryological evidence of TF interactions also exists. The cardiac phenotype was more severe in Nkx2-5(-/-)/Hand2(-/-) embryonic mice than in either gene knockout alone, and these embryos failed to generate a ventricular chamber (Yamagishi et al., 2001). Similarly, co-deletion of Tbx5 and Nkx2-5 resulted in a more severe phenotype than that observed for single mutants, and transcriptomics of single and double mutants revealed both transcriptional synergy and antagonism at downstream target genes (Luna-Zurita et al., 2016). In the same study, physical interaction of TFs at the Nppa promoter sequence was supported by x-ray crystallization, and chromatin immunoprecipitation sequencing (ChIP-seq) studies revealed the existence of novel DNA motifs modulating synergistic and non-synergistic gene activation (Luna-Zurita et al., 2016). Thus, physical interactions between the core cardiac TFs are widespread, and these lead to activation of specific target genes.

2.3.12 Transcription factor-based reprogramming of stromal cells to cardiomyocytes

Experiments conducted over 30 years ago revealed that overexpression of a single TF, Myoblast determination protein 1 (MyoD), could reprogram embryonic fibroblasts to skeletal myoblasts (Davis et al., 1987). Interestingly, reprogramming was enhanced by co-transfection with MEF2 TFs (Molkentin et al., 1995). Over two decades after those experiments, it was discovered that the overexpression of GATA4, MEF2C, and TBX5 TFs reprogrammed cardiac and dermal fibroblasts into spontaneously beating cardiomyocyte-like cells (Ieda et al., 2010). This approach was also successful *in vivo*, and studies suggested improved reprogramming efficiency and functional improvements after MI in mouse models (Qian et al., 2012; Song et al., 2012). Direct cardiac reprogramming was extended to human fibroblasts, though it required an alteration of the protocols to include a different set of TFs and/or microRNAs (Fu et al., 2013; Nam et al., 2013). Interestingly, reprogramming of mouse embryonic fibroblasts with GATA4, MEF2C, TBX5, and HAND2 was

shown to result in a heterogeneous mixture of cardiomyocytes with atrial, ventricular, and pacemaker cell electrophysiological phenotypes (Nam et al., 2014).

Several aspects of reprogramming hint at its molecular mechanisms. TF stoichiometry was key to ensuring reprogramming of fibroblasts to cardiomyocytes, and reprogramming efficiency was optimal when there were higher levels of MEF2C than GATA4 and TBX5 (Wang et al., 2015). Additionally, it was shown that multiple TFs needed to be expressed at a single cell level in order for cell fate transition to the cardiomyocyte lineage to take place (Zhang et al., 2019). Chromatin regulation also appears to be important to cell reprogramming. shRNA screening revealed that polycomb repressor protein Bmi1 was an inhibitor of reprogramming, and when depleted it allowed reprogramming of fibroblasts to cardiomyocytes via two transcription factors: MEF2C and TBX5 (Zhou et al., 2016). An overexpression screen of kinases revealed that protein kinase B (Akt1) stimulated reprogramming of fibroblasts to mature cardiomyocytes by GATA4, HAND2, MEF2C, and TBX5 (Zhou et al., 2015). In a separate overexpression study involving screening of a cDNA library, the transcription factor zinc finger protein 281 (ZNF281) was found to enhance the efficiency of reprogramming by interacting with GATA4 at cardiac enhancer elements (Zhou et al., 2017). In addition to illustrating the role of TFs as master regulators of cell fate, mechanisms of direct lineage reprogramming give insight into the function of cardiac transcriptional networks.

2.3.13 Chromatin accessibility and chromatin-modifying enzymes

In addition to TFs, genetic studies have implicated several chromatin-modulating enzymes in cardiac development. In a landmark study, Mammalian Chromatin-Remodeling Complex BRG1-Associated Factor 60C (BAF60C) was shown to be a cardiac-specific subunit of the SWI/SNF ATP dependent chromatin remodeling complex (BAF), and when deleted resulted in defects in cardiac morphogenesis, purportedly via altered access of developmental cardiac TFs to regulatory DNA elements (Lickert et al., 2004). Cardiac-specific deletion of Hdac1 and Hdac2 revealed redundant roles for these genes, but when co-deleted resulted in neonatal lethality due to mis-regulated gene expression programs, arrhythmias, and dilated cardiomyopathy (Montgomery et al., 2007). Deletion of the enhancer of zeste 2 polycomb repressive complex 2 subunit (Ezh2) using Nkx2-5-Cre resulted in compact myocardial hypoplasia, hypertrabeculation, and ventricular-septal defects (He et al., 2012). However, loss-of-function of the same gene using Mef2cAHF-Cre, a marker of the anterior heart field, resulted in a hypertrophied right ventricle and a failure to adequately express gene programs associated with cardiac progenitors (Delgado-Olguín et al., 2012). Hdac3 was also necessary for the differentiation of neural crest cells into the smooth muscle lineage composing the aortic arches (Singh et al., 2011). Thus, chromatin-modifying enzymes can underlie cardiac-specific phenotypes.

2.3.14 Role of fetal transcription factors in the maintenance of cardiac homeostasis in the adult

The expression of numerous developmental TFs persists into adulthood, and these factors are critical to postnatal cardiac function. Genetic approaches have further revealed the role of core cardiac TFs in the adult, and the relationship between developmental and adult phenotypes in loss- and gain-of-function mice is displayed in Table 4. For instance, postnatal deletion of Gata4 resulting in reduced (50%), but not total absence of Gata4 protein caused cardiomyocyte hypoplasia and pressure-overload induced apoptosis (Springer et al., 2006). Cardiomyocyte-specific deletion of Gata4 caused heart failure in mice, in addition to impaired hypertrophy after pressure overload and/or exercise (Oka et al., 2006). In addition to Gata4, conditional deletion of Mef2a in the postnatal heart led to dysregulation of genes in the atria and ventricles (Medrano & Naya, 2017). Furthermore,

cardiomyocyte-specific deletion of Srf in the adult heart led to reduced left ventricular contractility, dilated cardiomyopathy, and death from heart failure (Parlakian et al., 2005).

Cardiac TFs also regulate the electrical activity of the heart. For instance, Tbx5 deletion in adult mice caused sustained atrial fibrillation due to defects in Ca^{2+} handling (Nadadur et al., 2016). Similarly, specific deletion of Tbx5 within the ventricular conduction system induced arrhythmias and death (Arnolds et al., 2012). Importantly, a human loss-of-function mutation in TBX5 was shown to have decreased transcriptional activity, decreased synergy with NKX2-5, and increased susceptibility to atrial fibrillation (Guo et al., 2016). Similarly, deletion of Tbx5 in adult mice resulted in atrial fibrillation via the altered expression of atrial fibrillation-associated target genes (Nadadur et al., 2016). In addition to loss-of-function mutations, a gain-of-function Tbx5 mutation which increases TBX5 DNA binding was identified, and patients with this mutation had both congenital heart disease and atrial fibrillation, suggesting that excessive TBX5 activity is also deleterious in humans due to altered regulation of genes defining the heart's electrical activity (Postma et al., 2008). Nkx2-5 also regulated conduction system function, as overexpression of pathogenic variants of NKX2-5 which have reduced DNA binding in adult mouse hearts led to atrioventricular conduction defects and downregulation of Connexins 40 and 43 (Kasahara et al., 2001).

In addition to electrical activity, evidence exists of the regulation of blood pressure by developmental cardiac TFs. In the largest study to date of genes regulating blood pressure, including over 1 million participants, single nucleotide polymorphisms (SNPs) in developmental genes MEF2A, T-box transcription factor 20 (TBX20), transcription factor 4 (TCF4), meis homeobox 1 (MEIS1), cholecystokinin B receptor (CCKBR), and transforming growth factor beta receptor 3 (TGFBR3) (among other genes) were identified as affecting blood pressure levels (Evangelou et al., 2018). Additionally, single nucleotide polymorphisms (SNPs) at the Tbx5-Tbx3 locus have appeared in two genome-wide association studies seeking to identify genes regulating blood pressure (Fedorowski et al., 2012; Levy et al., 2009). Furthermore, adult mice with Tbx5 haploinsufficiency have altered diastolic function (Zhu et al., 2008). Thus, many of the same transcription factors regulating cardiogenesis also regulate physiological function in the adult.

2.3.15 Re-activation of fetal transcription factors in the adult heart during pathological processes

Abundant evidence exists that in the diseased, adult heart there is a re-emergence of fetal TF activity, and that these TFs regulate compensatory cellular processes in the diseased heart, such as hypertrophy (Oka et al., 2007). In this line, several studies have indicated that the mRNA and protein levels of cardiac TFs are modified in response to pathological stimuli in the adult heart. For instance, isoprenaline or phenylephrine infusion in adult mice led to upregulation of Nkx2-5 and Gata4 mRNA 3, 7, and 14 days post-treatment (Saadane et al., 1999). During cardiac hypertrophy following right ventricular pressure overload in cats, Nkx2-5 mRNA was also upregulated (Thompson et al., 1998). Global RNA-seq experiments comparing mRNA profiles of fetal rat hearts and samples from hearts with pressure-induced overload and hypertrophy showed remarkable overlap in mRNA splicing isoforms between the two conditions, suggesting that the fetal mRNA splicing mechanism is induced during the pathological response (Ames et al., 2013). In right ventricular pressure overload induced by banding of the pulmonary artery in rats, the core cardiac TFs Gata4, Nkx2-5, Mef2, and Hand2 were upregulated in the right ventricle (Bär et al., 2003). Similar to other core cardiac TFs, Hand2 is upregulated by ET-1 signaling (Charité et al., 2001).

Additional work has indicated that transcription factor activity is increased in the disease setting, often via post-translational modifications. Notably, the cardiac master regulator Gata4 displayed increased activity in response to pressure overload in adult rats (Herzig et al., 1997). This effect was blocked by endothelin receptor antagonists, suggesting that increased GATA4 activity is due to increased ET-1 in the pressure overload model (Hautala et al., 2001). Indeed, ET-1 was reported to cause phosphorylation of GATA4, modifying its transcriptional activity (Kitta et al., 2001). In a landmark study, overexpression of either Gata4 or Gata6 resulted in cardiomyocyte hypertrophy *in vitro* and *in vivo*, providing further evidence of the role of GATA factors in the pathological response (Liang et al., 2001). Generation of mutant mice with an amino acid substitution preventing GATA4 phosphorylation resulted in mice which failed to induce cardiac hypertrophy following phenylephrine infusion (van Berlo et al., 2011). Curiously, these mice were also more susceptible to heart failure (van Berlo et al., 2011).

MEF2 factors are also instrumental to adult heart disease response and pathological hypertrophy. Similar to Gata4, Mef2c is also a downstream target of endothelin signaling (Hu et al., 2015). Overexpression of Mef2a and Mef2c in transgenic mice resulted in altered outward potassium currents and dilated cardiomyopathy (Xu et al., 2006). However, the direct Mef2 targets which resulted in this phenotype were not described (Xu et al., 2006). Mef2a and Mef2d are the predominant Mef2 factors in the adult heart, and conditional deletion of Mef2d resulted in resistance to pathological hypertrophy, whereas overexpression of Mef2d induced pathological hypertrophy (Kim et al., 2008). Additionally, an inherited mutation has been described in Mef2a which leads to coronary artery disease (Wang et al., 2003). MEF2 is also dysregulated in pulmonary artery endothelial cells of patients with pulmonary arterial hypertension, and this leads to altered transcription of genes involved in vascular homeostasis (Kim et al., 2015). Exercise also appears to modulate MEF2 activity. Mef2 activity in skeletal muscle increased during endurance exercise, and this increase in activity was via calcineurin signaling (Wu et al., 2001).

Overexpression studies have indicated effects of fetal TFs in the heart. Cardiomyocyte-specific overexpression of Srf led to the development of hypertrophic cardiomyopathy (Zhang et al., 2001). Overexpression of the ventricle-specific Hey2 TF attenuated the hypertrophic response to phenylephrine in vitro and in vivo, potentially via inhibition of GATA4 activity (Xiang et al., 2006). Additionally, microRNAs also play a role in cardiovascular disease processes. Inhibition of the Let-7 microRNA, which regulates maturation of embryonic cardiomyocytes, improves postinfarction myocardial function (Tolonen et al., 2014). Another microRNA, miR-34a, is induced in the aging heart and causes age-associated cardiomyocyte cell death via regulation of Protein Phosphatase 1 Regulatory Subunit 10 (PPP1R10) (Boon et al., 2013). Interestingly, some members of the core cardiac transcription factor network are downregulated following disease stimuli. HAND factors were downregulated in response to hypertrophic stimuli in the rat, suggesting they are part of the normal, rather than pathological cardiac gene regulatory network (Thattaliyath et al., 2002). Similarly, in the border zone of the infarcted left ventricle, MEF2 transcriptional activity decreases and is replaced by activator protein 1 (AP-1) transcriptional activity (van Duijvenboden et al., 2019).

The role of the core cardiac TFs in cardiac regeneration has also been investigated. During zebrafish heart regeneration, proliferating cardiomyocytes were shown to be derived from a Gata4+ lineage (Kikuchi et al., 2010). In a separate study, core cardiac transcription factors Gata4, Hand2, Nkx2-5, Tbx5a, and Tbx20 were re-activated in the infarcted zebrafish heart prior to regeneration (Zhang et al., 2013). Gata4 overexpression also improved the regenerative capacity of the neonatal mouse after myocardial infarction (Malek et al., 2017). In mice lacking the left/right asymmetry master regulator

Pitx2, neonatal heart regeneration was impeded following apex resection, purportedly via the transcriptional regulation of metabolic genetic programs (Tao et al., 2016). Thus, the effects of re-activation of cardiac TFs on the diseased heart is context-dependent. Additionally, abundant evidence exists that the transcriptional mediators of cardiac development are also the mediators of adult heart diseases.

2.4 Chamber-specific markers of therapeutic relevance – transcriptional targets of the core cardiac transcription factors as mediators of cardiac diseases

gene	functional class	cardiomyocyte subtype	stage at which subtype-specific expression begins	gain- and/or loss-of-function phenotype in mice	Known transcriptional regulators	citations
Cntn2	cell adhesion molecule	cardiac conduction system	adult (embryonic expression unpublished)	-	-	Pallante et al., 2010
Cx40	gap junction	atrial cardiomyocytes, conduction system	adult (pan-cardiac during embryogenesis)	Germline deletion leads to lower conduction velocity, increased arrhythmias, conduction system defects, alters action potential propagation in the right atria.	Tbx5 (repressor) Gata4, Nkx2-5	Bagwe et al., 2005; Gros et al., 1994; Gros & Jongsma, 1996; Gourdie et al., 1993; Kirchhoff et al., 1998
Hcn4	ion channel	sinoatrial node	neonatal->adulthood (during embryogenesis, in first heart field mesoderm and then progressively restricted).	Germline or cardiomyocyte-specific results in absence of pacemaker current and E9.5-E11.5 lethality.	Mef2 factors	Garcia-Frigola et al., 2003; Kuratomi et al., 2009; Stieber et al., 2003
Kcna5	ion channel	atrial cardiomyocytes	-	-		Feng et al., 1997
Myl2	sarcomeric machinery	ventricular cardiomyocytes	E8->adulthood	Germline deletion results in E12.5 lethality due to impeded sarcomeric assembly which leads to dilated cardiomyopathy. Alters contractile properties of atria in overexpression studies.		Buck et al., 1999; Chen et al., 1998; Pawloski-Dahm et al., 1998

Table 5 (continued on next page). Cardiomyocyte subtype-specific markers delineating anatomical substructures of the heart. Markers representing cells of the atria, ventricle, and conduction system show varying temporal onset of specificity. Functional studies reveal integral roles for many of these proteins in the function of the heart. Those with known transcriptional regulators are also included, though detailed studies for regulation of many of these markers do not exist. Contactin-2 (Cntn2), Connexin 40 (Cx40), Hyperpolarization activated cyclic nucleotide gated potassium channel 4 (Hcn4), Potassium voltage-gated channel subfamily A member 5 (Kcna5), Myosin light chain 2 (Myl2), Myosin light chain 7 (Myl7), Natriuretic peptide A (Nppa), Sarcolipin (Sln), Slow myosin heavy chain 3 (SMyHC3).

gene	functional class	cardiomyocyte subtype	stage at which subtype-specific expression begins	gain- and/or loss-of-function phenotype in mice	Known transcriptional regulators	citations
Myl7	sarcomeric machinery	atrial cardiomyocytes	E12->adulthood (pan-cardiac E8-E11).	Germline deletion leads to impaired atrial contractiliy and embryonic lethality E10.5-11.5	Hey2	Huang et al., 2003; Koibuchi & Chin, 2007; Kubalak et al., 1994
Nppa	peptide hormone	atrial cardiomyocytes, traneculated ventricular cardiomyocytes	neonatal-> adulthood (pan-cardiac during embryogenesis with higher expression in ventricle).	Germline deletion results in hypertension and increased cardiac hypertrophy in response to volume overload.	Gata4, Nkx2-5, Tbx5	Bruneau et al., 2001; Durocher et al., 1996; Durocher et al., 1997; John et al., 1995; Mori et al., 2004; Zeller et al., 1987
Sln	calcium handling	atrial cardiomyocytes (extra-cardiac expression in all striated muscle except ventricular muscle).	E12->adulthood	Germline deletion results in increased cardiac contracility, but long term effects are increased fibrosis and occurrence of arrythmias. Codeletion with phospholamban leads to decreased contractility and hypertrophy. Downregulated during chronic atrial fibrillation in humans.	-	Babu et al., 2007; Minamisawa et al., 2003; Shanmugam et al., 2011; Uemura et al., 2004; Xie et al., 2012
SMyHC3 (quail)	sarcomeric machinery	atrial cardiomyocytes from early embryogenesis onwards (in mice)	E8.25->adulthood	-	Irx4	Nikovits et al., 1996; Wang et al., 1996; Xavier-Neto et al., 1999

Despite the known importance of core cardiac TFs in development and disease, little is known about the precise transcriptional targets which give rise to the phenotypes arising from depletion or overexpression of the TFs themselves. A more detailed understanding of these targets could provide new therapeutic avenues for those suffering from congenital or adult-onset heart diseases. As a detailed description of all transcriptional targets would be cumbersome, this section will focus on the transcriptional targets with known chamber-specific expression patterns which confer chamber- and subtype-specific function. Gain- or loss-of-funtion phenoptypes for some of these subtype specific genes are summarized in Table 5. Examination of this transcriptional regulation partially explains the overlap in phenotypes of TFs in the etiology of congenital heart disease, cardiac arrhythmias, cardiac regeneration, and heart failure. Importantly, examination of the different gene expression patterns of

atrial and ventricular cardiomyoyctes is also illustrative of physiological differences between cardiomyocyte subtypes (Figure 5).

2.4.1 Sarcomeric markers of chamber myocardium – effectors of growth, form, and function

Contractile properties of the atria and ventricles differ, as atria have a higher maximum shortening velocity, generate less active tension, and have a lower resting tension (Ng et al., 2010). Additionally, ventricular muscle was shown to generate more force per ATP consumed and to be nine times slower than atrial muscle (Narolska et al., 2005). Cardiac myosin is a mediator of contractile force and is composed of two heavy chains and four light chains, and atrial or ventricular cardiac myosin light chain isoforms were identified as determining the varying force-generating capacity of myosin (Ng et al., 2010; Yamashita et al., 2003). Importantly, myosin regulatory light chains are differentially expressed across the atria and ventricles, and this has led to an improved understanding of cardiac chamber formation and cardiomyocyte-subtype differentiation programs. Myosin regulatory light chain 2 (Myl2) was initially cloned from rat cDNA prepared from ventricular cardiomyocyte lysates, and a 250bp promoter fragment was shown to confer cardiomyocyte-specific expression (Henderson et al., 1989). Myl2 mRNA expression was further characterized using *in situ* hybridization and shown to be expressed in the developing ventricle and outflow tract, but mRNA was absent from the developing atria (Franco et al., 1999; O'Brien et al., 1993). Deletion of Myl2 resulted in embryonic lethality at E12.5 due to defects in sarcomere assembly which caused embryonic dilated cardiomyopathy (Chen et al., 1998). Strikingly, overexpression of the ventricular isoform (Myl2) in the whole heart altered the mechanical properties of the atria so that they resembled the ventricle, in addition to increasing the cycling rate (Buck et al., 1999; Pawloski-Dahm et al., 1998). Regulators of Myl2 were observed to include protein kinase C and the α-adrenergic agonist Phenylephrine, and these pathways also regulated the Nppa promoter (Knowlton et al., 1991; Lee et al., 1988; Shubeita et al., 1992). Genetic manipulation of the Myl2 gene has been used to study ventricular formation and biology, first using a transgenic mouse line in which Myl2 promoter sequences were used to drive a luciferase reporter (Lee et al., 1992). Myl2 was also expressed during mESC differentiation to cardiomyocytes in embryoid bodies, suggesting that *in vitro* models can be used to study ventricular

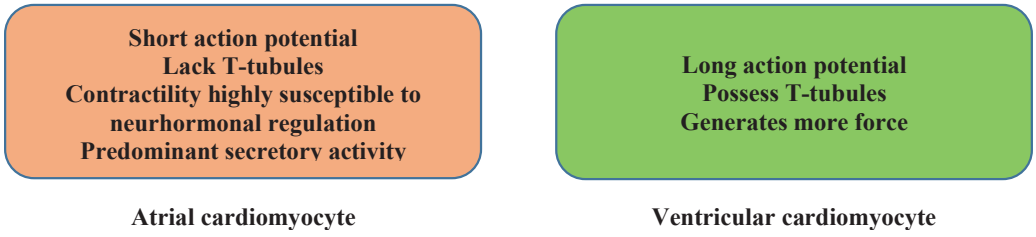

Figure 5. Molecular and functional differences between atrial and ventricular cardiomyocytes.

formation (Miller-Hance et al., 1993). To this end, several reporter mESC lines have been generated using either Myl2 promoter fragments or Myl2-cre gene targeting to study ventriculogenesis *in vitro* (Lee et al., 2012; Moore et al., 2004; Müller et al., 2000).

Myosin light chain 7 (Myl7) displayed a pan-cardiac expression pattern during early cardiogenesis (E8-D12), and its expression was restricted to the atrium from E12 into adulthood (Kubalak et al., 1994). Similar to its ventricular counterpart Myl2, germline deletion of Myl7 led to defects in atrial contraction and embryonic lethality at E10.5-E11.5 (Huang et al., 2003). In chick embryos, the retinoic-acid target gene atrial-specific myosin heavy chain (AMHC1) was observed to be expressed

in only the developing atria, but in the presence of exogenous retinoic acid it was expressed throughout the heart, providing further evidence of the role of retinoic acid signaling in anterior-posterior patterning (Yutzey et al., 1994). In quail embryos, the slow myosin heavy chain 3 (SMyHC3) gene was observed to have pan-cardiac expression during early embryogenesis, but mRNA was later restricted to the atria (Nikovits et al., 1996; Wang et al., 1996). Importantly, a quail regulatory sequence of the SMyHC3 gene was used to mark the atrial lineage in transgenic mice, showing mostly restricted expression in atrial regions during embryogenesis and in the adult (Xavier-Neto et al., 1999). This well-characterized promoter sequence was activated by Gata4, but not Mef2c or Nkx2-5 (Wang et al., 1998). In the atria, SMyHC3 expression was activated by retinoic acid signaling, whereas in ventricular cardiomyocytes it was repressed by an inhibitory complex involving the ventricular-specific Irx4 transcription factor, the vitamin D response element, and Rxrα (Wang et al., 2001). Thus, atrial and ventricular isoforms of sarcomeric genes give insight into differences in atrial and ventricular contraction, in addition to gene regulatory networks governing specific atrial and ventricular activity.

2.4.2 Chamber-specific gap junctional proteins and cell adhesion molecules

Gap junctions in the heart allow for the intercellular conduction of ions, metabolites and second messengers between cardiomyocytes, and these gap junctions were shown to be encoded by Connexin 40 (CX40) and connexin 43 (CX43) (Gros & Jongsma, 1996). Importantly, Cx40 was shown to be expressed in atrial cardiomyocytes and the conduction system, but it was absent from ventricular cardiomyocytes (Gourdie et al., 1993; Gros et al., 1994). Germline deletion of Cx40 resulted in lower conduction velocities in both atria and ventricles and also led to increased arrhythmias (Kirchhoff et al., 1998). A separate loss-of-function study found that loss of Cx40 led to atrioventricular and bundle branch block, implicating its specific role in the conduction system (Simon et al., 1998). An additional follow-up study showed that deletion of Cx40 impeded propagation of the action potential in the atria, specifically on the right side of the heart (Bagwe et al., 2005). Cx40 was also shown to be necessary for proper function of the sinoatrial node (Leaf et al., 2008). Thus, the predominant role of gap junctions is in the propagation of the action potential which induces cardiac contraction. Interestingly, Cx40 was shown to be regulated at the transcriptional level by core cardiac TFs TBX5, GATA4, and NKX2-5 (Arnolds et al., 2012; Linhares et al., 2004). In addition to regulating Cx40, NKX2-5 was also shown to be a transcriptional repressor of Cx43 (Kasahara et al., 2003). Finally, Contactin-2 (Cntn2), which is a cell adhesion molecule with known functions in the mammalian nervous system, was specifically expressed within the conduction system of the adult heart (Pallante et al., 2010).

2.4.3 Chamber-specific ion channels underlying differential manifestations of the action potential across cardiomyocyte subtypes

The action potential is initiated in the sinoatrial node and transmitted to all cells of the heart, but has noticeably different shapes in cardiomyocyte subtypes due to differential expression of ion channels. These ion channels open and close in response to changes in membrane potential, and differential expression of these channels are responsible for the varying action potential shapes between atrial, ventricular, and conduction system cells. The cardiac action potential is characterized by phases 0-4, representing changes in the membrane potential of the cell with respect to time (Liu et al., 2016). Importantly, atrial cardiomyocytes from both large and small mammals contain an ultra-rapid outward K+ current which is absent in ventricular cardiomyocytes, called I_{kur} (Boyle et al; 1991; Wang et al., 1993). This current was shown to be encoded by the atrial-specific Potassium voltage-

gated channel subfamily A member 5 (Kcna5) gene and to be responsible for the shorter action potential which is characteristic of atrial cardiomyocytes (Feng et al., 1997). Interestingly, the parasympathetic nervous system controls heart function via the release of acetylcholine, and the acetylcholine-activated K+ current ($I_{K, Ach}$), encoded by the Potassium inwardly rectifying channel subfamily J member 3 (Kcnj3) and Kcnj5 genes, was shown to be expressed in the atria, sinoatrial node, and atrioventricular node, but not the ventricular myocardium (Liu et al., 2016). Thus, neurohormonal regulation differs across cardiac chambers and cardiomyocyte subtypes. Additional Ca^{2+} and Ca^{2+}-activated K+ currents were also shown to be present in atrial, but not ventricular cardiomyocytes (Liu et al., 2016). Conduction system cells, which have strikingly different action potential shapes, also possess subtype-specific ion channels. Importantly, sinoatrial nodal cells were shown to possess hyperpolarization-activated cation currents (pacemaker currents) encoded by hyperpolarization activated cyclic nucleotide gated potassium channels (HCN) Hcn1, Hcn2, and Hcn4; which themselves displayed conduction system-specific gene expression patterns (Liu et al., 2016).

Regulation of ion channel gene expression by cardiac TFs might explain both genetic and epigenetic aetiologies of arrhythmias in congenital and adult heart disease. A gradient of Irx5 expression was shown to regulate the transcription of Kv4.2, and deletion of the Irx5 transcription factor led to increased arrhythmias (Costantini et al., 2005). Furthermore, the sodium/calcium exchanger (Ncx1) was shown to be a transcriptional target of NKX2-5 and SRF in adult and neonatal cardiomyocytes (Müller et al., 2002). Hcn4 was identified via expression studies as a marker of the cardiac crescent which was later restricted to the sinoatrial node (Garcia-Frigola et al., 2003). Additionally, Hcn4 was identified as a transcriptional target of MEF2 TFs (Kuratomi et al., 2009). Deletion of Tbx5 from the ventricular cardiac conduction system resulted in arrhythmias and reduced survival via altered transcription of channel proteins sodium voltage-gated channel alpha subunit 5 (Scn5a) and the gap junction protein Cx40 (Arnolds et al., 2012). Indeed, Scn5a and sodium voltage-gated channel alpha subunit 10 (Scn10a) are transcriptional targets of both TBX3 and TBX5 (van den Boogaard et al., 2012). These studies give insight into the electrical defects observed upon deletion of cardiac TFs. A detailed understanding of the relationship between genetic underpinnings of arrhythmias could lead to improved targeted therapies. Additionally, chamber-specific ion channels serve as potential markers for cardiomyocyte subtypes and also drug targets in the treatment of chamber-specific arrhythmias.

2.4.4 Cell cycle genes regulated by core cardiac transcription factors governing cellular proliferation during heart growth

Development of the asymmetrical heart necessitates differential proliferation across the cardiac chambers. This is controlled by cardiac gene regulatory networks, which in turn regulate the transcription of cell cycle genes. Recent work on cell cycle targets has led to an improved understanding of the specific mechanisms through which TFs exert changes in embryonic cardiac morphology. For instance, cell division control protein 42 (Cdc42) deletion in embryonic cardiomyocytes resulted in right ventricular hyperplasia, suggesting that Cdc42 is a major effector of embryonic cardiomyocyte proliferation (Liu et al., 2017). GATA4 was shown to be a transcriptional activator of cell cycle genes cyclin D2, cyclin A2, and cyclin dependent kinase 4 (Cdk4) in the right ventricle, thereby regulating the proliferation of ventricular cardiomyocytes (Rojas et al., 2008). Additionally, GATA4 and TBX5 have been shown to regulate cell cycle genes in second heart field progenitor cells (Xie et al., 2012; Zhou et al., 2017). Thus, core cardiac TFs regulate cell cycle targets

and cellular proliferation in both multipotent CPs and differentiated cardiomyocytes, and this results in organ-level morphological changes.

2.4.5 Secreted peptides with endocrine, autocrine and paracrine functions as biomarkers of adult disease

Nearly forty years ago, it was observed that delivery of atrial, but not ventricular extract to non-diuretic rats resulted in large increases in both sodium and chloride excretions and urine volume, suggesting that the mammalian atria possessed unidentified, secreted, natriuretic-inducing hormones (de Bold et al., 1981). Additionally, atrial extracts were shown to act as relaxants of vascular smooth muscle cells, indicating a system by which the atria of the heart senses and then regulates total fluid volume and blood pressure via natriuresis and vasodilation (Currie et al., 1983). The active peptide in atrial extract exerting these effects was determined to be atrial natriuretic factor, and its mRNA (encoded by the Nppa gene) was detected in both rat atria and ventricles (Gardner et al., 1986; Nemer et al., 1986; Seidah et al., 1984; Zivin et al., 1984). Additionally, expression was observed to be pan-cardiac during initial heart formation prior to restriction to the atria and trabeculated ventricle by neonatal stages (Zeller et al., 1987). Importantly, this gene was activated in ventricular tissue following acute myocardial infarction (Galipeau et al., 1988). In a landmark study, the Nppa promoter sequence was shown to be co-activated by cardiac TFs NKX2-5 and GATA4 (Durocher et al., 1996; Durocher et al., 1997). Additionally, Tbx5 deletion resulted in reduction of serum atrial natriuretic peptide (ANP) levels (Bruneau et al., 2001). Another T-box factor, TBX2, formed a repressive complex with NKX2-5 at the Nppa promoter in the atrioventricular canal, reducing its expression (Habets et al., 2002). Importantly, different regulatory regions of Nppa were shown to regulate homeostatic vs hypertrophic transcription of Nppa mRNA (Knowlton et al., 1995). Indeed, it was later discovered that the proximal promoter sequence of Nppa does not contain the necessary regulatory elements for ventricular re-activation of Nppa in heart disease models (Horsthuis et al., 2008).

Natriuretic peptide B (Nppb) has similar embryonic expression patterns to Nppa, but unlike Nppa it is not downregulated in the ventricles postnatally and maintains pan-cardiac expression (Cameron et al., 1996). However, injury has been reported to induce chamber-specific transcriptional responses. Nppb mRNA is increased in the right atria following induction of pressure in perfused rat hearts, suggesting that increased serum brain natriuretic peptide (BNP) is due to increased transcriptional activity (Magga et al., 1997). In a dog congestive heart failure model, Nppa mRNA was upregulated in both left atria and left ventricles, though the increases in the left ventricles were more pronounced (Luchner et al., 1998). Interestingly, ET-1 and Angiotensin antagonists blocked atrial, but not ventricular Nppb transcriptional activation in spontaneously hypertensive rats, suggesting distinct chamber-specific regulation of Nppb in the ventricles (Magga et al., 1997). Furthermore, GATA4 and NKX2-5 were shown to be principally responsible for the mechanical-stretch induced activation of the Nppb promoter (Pikkarainen et al., 2003). Similarly, two-weeks of angiotensin II infusion increased transcription of Nppb mRNA, and GATA4 binding sites were necessary for this increase (Majalahti et al., 2007).

Other important secreted proteins have also shown transcriptional regulation by Gata4. Gata4 was shown to increase the transcription of the endothelin-1 (ET-1) gene in neonatal rat cardiomyocytes (Morimoto et al., 2000). As Gata4 is also activated by ET-1, this indicates that a positive feedback loop at the transcriptional level may exist in pathological circumstances (Hautala et al., 2001). The vascular endothelial growth factor A (VEGF-A) is a transcriptional target of Gata4, and

cardiomyocyte-specific overexpression of Gata4 in the adult heart leads to increased angiogenesis, purportedly via increased secretion of VEGF from cardiomyocytes and/or increased stem cell recruitment (Heineke et al., 2007; Rysä et al., 2010). Thus, the transcriptional targets of core cardiac TFs include secreted factors which can induce organ- and organismal-level changes in function.

2.4.6 Chamber-specific regulators of intracellular Ca^{2+}

Cardiac contractility is controlled by excitation-contraction coupling, which is itself a function of an increase in intracellular Ca^{2+} levels induced by the opening of voltage-gated Ca^{2+} channels as the action potential initiated by the sinoatrial node spreads throughout the heart (Luo & Anderson, 2013). Though a deep exploration of Ca^{2+} machinery is beyond the scope of the current thesis, an investigation into chamber-specific differences of Ca^{2+} handling properties is illustrative, as it reflects differences in contractile properties across the chambers of the heart. Most strikingly, ventricular cardiomyocytes possess long invaginations into the cell covered with voltage-gated Ca^{2+} channels called transverse-tubules (T-tubules), and these allow a synchronized increase in intracellular Ca^{2+} in response to an action potential (Bootman et al., 2006). Interestingly, atrial cardiomyocytes lack a network of T-tubules, thus increases in intracellular Ca^{2+} depend only on voltage-gated Ca^{2+} channels which are located on the periphery of cells (Bootman et al., 2006). This Ca^{2+} signal must be further transported to the contractile machinery in order to initiate atrial muscle contraction (Bootman et al., 2006). The functional consequence of this difference is that the contraction of atrial cardiomyocytes is thought to be more dependent than ventricular cardiomyocytes on the concentration of Ca^{2+} in the sarcoplasmic reticulum, in addition to stimulation by inotropic agents (Bootman et al., 2006). Indeed, during basal conditions gradients of Ca^{2+} have been observed in atrial cardiomyocytes, and only via ET-1 treatment, beta-adrenergic receptor agonist treatment, or inositol 1,4,5-trisphosphate infusion was Ca^{2+} transmitted throughout the cell area of atrial cardiomyocytes (Mackenzie et al., 2004). These studies suggest that excitation-contraction coupling of atrial cardiomyocytes is more prone to regulation by external agonists. Indeed, defects in Ca^{2+} homeostasis are believed to underlie atrial fibrillation (Voigt et al., 2012).

In addition to the structural differences across the chambers of the heart, there are some reports of tissue-specific expression of Ca^{2+}-handling proteins governing intracellular Ca^{2+} levels. Phospholamban (PLN) is a 52 amino acid peptide which regulates the uptake of Ca^{2+} into the sarcoplasmic reticulum, thereby affecting myocardial contraction and relaxation (Davis et al., 1990; Edes & Kranias, 1987; Simmerman et al., 1986). In adult mice, Pln expression is upregulated in the ventricles compared to the atria, suggesting chamber-specific expression reflects divergent contractility across cardiac chambers (Koss et al., 1995). Interestingly, deletion of Pln in the murine model resulted in increased contractility due to enhanced Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity (Luo et al., 1994). Surprisingly, deletion of Pln rescued a genetic model of dilated cardiomyopathy (Minamisawa et al., 1999). In humans, mutations in the PLN gene have been shown to cause inherited forms of dilated cardiomyopathy (Schmitt et al., 2003).

In contrast to Pln, which is upregulated in the ventricles, the small peptide Sarcolipin (Sln) was expressed in the atria throughput embryogenesis and postnatally, and was downregulated in response to hypertrophic stimuli (Minamisawa et al., 2003). Additionally, Sln was shown to regulate activity of the SERCA2a Ca^{2+} pump, and Sln overexpression resulted in decreased cardiac contractility (Asahi et al., 2004). Interestingly, Sln was also shown to be abundantly expressed in fast-twitch skeletal muscle fibers (Minamisawa et al., 2003). Indeed, SLN protein was present in all striated muscle (including skeletal), except the ventricular myocardium (Babu et al., 2007). Deletion of Sln in mice

resulted in increased affinity of the SERCA pump for Ca^{2+}, thereby increasing Ca^{2+} uptake and atrial contractility (Babu et al., 2007). Long term effects of loss of Sln included increased atrial fibrosis and increased frequency of arrhythmias during aging (Xie et al., 2012). Additionally, Sln was involved in SERCA-mediated muscle thermogenesis and metabolism (Bal et al., 2012). Interestingly, deletion of both Pln and Sln resulted in decreased contractility and onset of cardiac hypertrophy, distinct from the phenotypes resulting from either peptide alone (Shanmugam et al., 2011).

Oftentimes, mutations causing loss- or gain-of-function of core cardiac TFs lead to misexpression of Ca^{2+} handling proteins, thereby generating defects in Ca^{2+} handling and arrhythmias. For instance, deletion of the Tbx5 transcription factor in adult mice led to downregulation of Ca^{2+} handling proteins Ryanodine receptor 2 (Ryr2), ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2 (Atp2a2), and sarcolipin (Sln), in addition to upregulation of Pln (Dai et al., 2019). Interestingly, when Pln was depleted, atrial fibrillation in Tbx5 mutant mice was eliminated, demonstrating the potential to treat genetic diseases caused by TFs via the modulation of specific downstream target proteins (Dai et al., 2019). Collectively, these studies have demonstrated that Ca^{2+} handling properties across cardiac chambers are distinct, and some molecular targets and their transcriptional regulators have been identified. These might hold great promise for the regulation of cardiac function.

Recent work on store operated Ca^{2+} entry (SOCE) has given insight into chamber-specific regulation of Ca^{2+} during cardiac development and function. SOCE occurs when intracellular Ca^{2+} levels are low, and this process also occurs in cardiomyocytes (Uehara et al., 2002). This is mediated by stromal interaction molecule 1 (STIM1), which is a sensor for intracellular Ca^{2+} levels and an activator of store-operated Ca^{2+} entry (SOCE) (Rosenberg et al., 2019). Interestingly, STIM1 was shown to bind to PLN and indirectly activate SERCA2 activity in cardiomyocytes (Zhao et al., 2015). STIM1 was further repressed by PLN, and this was believed to inhibit SOCE (Seth et al., 2012). Interestingly, in the adult mouse heart STIM1 expression was restricted to the sinoatrial node, and it was there thought to regulate the sinoatrial node's characteristic repolarization phase and automaticity (Rosenberg et al., 2019; Zhang et al., 2015). Surprisingly, Stim1 knockdown inhibited phenylephrine-induced hypertrophic growth of neonatal rat cardiomyocytes, providing a new potential pathway for the treatment of heart failure (Voelkers et al., 2010).

Importantly, Ca^{2+} holds two functions in the heart: regulation of contraction and regulation of intracellular signaling (Goonasekera & Molkentin, 2012; Molkentin, 2006). It is still unknown precisely how the two occur simultaneously, but Ca^{2+} signaling is integral to both cardiomyocyte function and cardiomyocyte hypertrophy. This occurs partially via the Ca^{2+}-dependent phosphatase calcineurin, which in turn affects NFAT and GATA4 activity (Molkentin et al., 1998). This signaling also has a chamber-specific role, as NFAT translocation to the nucleus (and activity) occurs in response to ET-1 or angiotensin II stimulation in adult atrial, but not ventricular cardiomyocytes (Rinne et al., 2010). Interestingly, external pacing of cells also increases NFAT activity, leading to the activation of the Nppb promoter (Tavi et al., 2004).

2.5 CCK as a physiological regulator

Recent work has indicated that hormones previously characterized in non-cardiac tissues mediate effects on cardiovascular function and in some cases exhibit cardiac expression patterns. For instance, expression of proCck mRNA and encoded CCK peptide have been reported in both pig and rodent hearts (Funakoshi et al., 1994; Goetze et al., 2015). Insight into its activity in other tissues might give hints to its function in the heart. Additionally, the tissue-specific regulators of proCCK at transcriptional and post-transcriptional levels could reveal its utility as a biomarker in different

contexts. Cholecystokinin (CCK) was initially identified as a secreted gut hormone which induced contraction of the gall bladder (Ivy & Oldberg, 1928). In a landmark study, exogenous CCK-8 injections in rats induced a marked reduction in food intake, grooming behavior, and sleep, indicating its role in the production of satiety (Gibbs et al., 1973). Furthermore, abdominal vagotomy demonstrated that this effect was due to binding to vagal nerves in the gut, not the brain (Smith et al., 1981). In addition to these effects, CCK-8 was shown to induce contraction of smooth muscle cells isolated from the stomachs of guinea pigs, and these effects were blocked by the CCK antagonist proglumide (Collins & Gardner, 1982). Importantly, CCK secretion in the digestive system was stimulated by dietary protein, but not by dietary fats, amino acids, or carbohydrates (Liddle et al., 1986). However, a recent study suggested that release of the CCK peptide from gastrointestinal cells also occurred in response to dietary fats and amino acids, and that this occurred due to an increase in intracellular Ca^{2+} (Wang et al., 2011). In a separate study, both dietary intake and gastrin-releasing peptide stimulated CCK peptide release (Lewis & Williams, 1990). Thus, CCK is a neurohormone, and its secretion is dependent on metabolic state.

2.5.1 proCCK as a cardiomyocyte marker

In the heart, proCCK was shown to be processed to a unique, sulfated form not previously described in other studies/tissues, suggesting that cardiac CCK could potentially bind to distinct receptors from intestinal or neuronal CCK (Figure 6) (Goetze et al., 2015). Plasma CCK levels increased post-myocardial infarction, and left non-infarcted ventricular myocardium displayed elevated levels of both proCck mRNA and protein at 4 and 6 weeks, but not 2 weeks, post-myocardial infarction (Dong

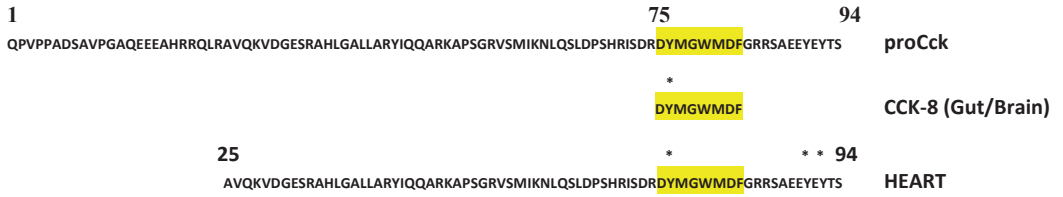

Figure 6. Processing of cholecystokinin peptide in the heart. Procholecystokinin peptide is processed intracellularly into unique forms. The most-studied CCK-8 form has been studied in nervous and the digestive systems. It was recently shown that a unique peptide form exists in cardiac tissue, though it is unknown what effects this has on receptor binding. * indicates sulfated residues. procholecystokinin (proCck), Cholecystokinin octapeptide (CCK-8).

et al., 2017). Plasma CCK levels correlated with plasma BNP levels, in addition to the left-ventricular end-systolic diameter (Dong et al., 2017). In a separate study in the porcine model, proCck was shown to be evenly expressed in the four chambers of the heart during neonatal and adolescent stages, but to be increased in the left atrium by adulthood (Goetze et al., 2018). Additionally, the authors observed decreased levels of peptide in the ventricles upon myocardial infarction, but observed no significant changes in the atria (Goetze et al., 2018). However, proCck was upregulated during both exercise and heart failure (Goetze et al., 2018). In a separate study, plasma CCK levels were predictive of mortality due to adverse cardiac events in elderly females (Goetze et al., 2016). Collectively, these results show complex regulation of proCck in cardiac chambers, potentially in response to hemodynamic changes in the heart.

2.5.2 Pharmacological studies reveal CCK as a modulator of cardiovascular function

Effects of exogenous CCK administration in rodents have been explored in several studies. Importantly, all studies included the shorter, processed forms prevalent in the brain: CCK-4 and CCK-8. Thus, they might not be fully reflective of effects occurring due to cardiac CCK, which is processed differently. In the first study of CCK action on the heart, CCK-8 induced bradycardia in anesthetized rats, indicating that like other neurohormones it is a regulator of heart rate (Marker & Roberts, 1988). As the same effects were observed on heart explants, the authors concluded that CCK acts on receptors endogenously expressed in the heart (Marker & Roberts, 1988). In a later study, it was shown that a lower dose of CCK (0.5 μg/kg i.v.) increased heart rate, whereas a higher dose (5.0 μg/kg, i.v.) caused 0.5-1 min bradychardia followed by 5-10 min tachycardia (Janssen et al., 1991). Thus, careful temporal monitoring is necessary to determine cardiovascular effects of CCK. Importantly, both doses also induced an increase in blood pressure (Janssen et al., 1991). In a separate study, it was confirmed that the observed effects of CCK were due to responses beyond the vagal nerve, purportedly via CCK receptors expressed in the heart (Kurosawa et al., 2001).

In human patients, injection of CCK-4 increased diastolic blood pressure and heart rate and also induced panic attacks (Bradwejn et al., 1992). A more detailed study showed that exogenous CCK-4 increased both heart rate and mean arterial blood pressure, and this effect was blocked by CCKBR antagonists (Fossa et al., 1997). In rats, CCK-33, but not CCK-8 and CCK-4 were shown to induce increases in systolic and diastolic blood pressure, in addition to heart rate (Wisniewska & Wisniewska, 1996). In the isolated heart, CCK-33 and CCK-8 caused increases in cardiac contraction, whereas heart rate was not affected, in contrast to other studies (Wisniewska & Wisniewska, 1996). In the pithed rat, sulfated CCK-8 induced bradycardia and an increase in mean arterial blood pressure, and this was inhibited by Cholecystokinin A receptor (CCKAR) antagonists, but not by CCKB receptor antagonists (Gaw et al., 1995). Experiments with adrenoreceptor antagonists phentolamine and guanethidine suggested that changes in mean arterial blood pressure (but not heart rate) occured due to activation of alpha-adrenoreceptors following CCK-8 treatment (Gaw et al., 1995). Importantly, alpha-adrenoreceptors themselves exist as a subfamily of receptors, with specific member expression in myocardium compared to coronary arteries, potentially exhibiting beneficial or deleterious effects (Jensen, Swigart, & De March et al., 2009; Jensen, Swigart & Laden et al., 2009). However, the precise relationship between CCK activity and specific alpha-adrenoreceptors is not known. The role of the CCKA receptor in mediating cardiovascular effects was further supported using CCKA receptor antagonists PD140548 and SR 27897B to counteract the effects of exogenously administered peptide (Bunting et al., 1997). In Otsuka Long-Evans Tokukshima Fatty rats, which lack the CCKA receptor and serve as a model of diabetes mellitus and obesity, exogenous CCK-8 did not exhibit characteristic decreases in heart rate, suggesting CCK-8 cardiovascular effects did not occur via CCKB receptors alone in this model (Kurosawa et al., 2001). These findings were confirmed in a later study (Kaczynska & Szereda-Przestaszewska, 2015). There is limited evidence of the effects of exogenous CCK in the disease setting. Gastrin, which binds the CCKB receptor, was reported to be cardioprotective in a rat model for myocardial ischemia, purportedly via the activation of downstream kinases (Yang et al., 2018). Recently, exogenous CCK-8 protected H9c2 cardiomyoblasts from angiotensin II-induced apoptosis (Wang et al., 2019). Collectively, these pharmacological studies of CCK indicate that it acts directly on the heart to regulate heart rhythm and potentially contractility. Furthermore, there is evidence that this can occur via both CCKA and CCKB receptors.

Pro-hypertensive effects of CCK have led to its exploration as a therapeutic agent for the treatment of hypotension. In an experimental model of haemorrhagic shock in rats, exogenous CCK-8 decreased

mortality from 100% to 0% by increasing blood pressure (Bertolini et al., 1986; Guarini et al., 1988). It was proposed that eliminating bradychardia induction with CCK-8 could potentially increase the beneficial effects further (Marker & Roberts, 1988). Exogenous CCK-8 improved cardiac function in endotoxic shock rats, purportedly via activation of CCKA and CCKB receptors in the myocardium (Zhao et al., 2005). This was confirmed in separate studies which also suggested that modulation of the inflammatory response is involved in the beneficial effects of exogenous CCK-8 (Saia et al., 2013; Saia et al., 2014). To date, there are no reports of using exogenous CCK or CCK receptor antagonists in studies in patients with cardiovascular disease. Thus, in the context of shock, pro-hypertensive effects of CCK might be beneficial. However, during heart failure these effects would be expected to be deleterious.

The regulation of the cardiovascular system by a digestive peptide might reflect increased circulatory requirements following ingestion of food. In dogs, vasodilation of arteries was induced by low concentrations of CCK in the duodenum and jejunum and by high levels of cholecystokinin in the heart, kidney, and spleen (Chou et al., 1977). In the Rainbow trout, it was shown that physiological levels of CCK increased gastrointestinal blood flow (Seth et al., 2010). Importantly, this effect appears to be due to direct actions of CCK on smooth muscle cells of the bulbus ateriosus (Seth et al., 2014). Therefore, the effects of digestive peptides on cardiovascular function appear to be related to the need for increased blood supply after eating.

2.5.3 Genetic studies reveal role of cholecystokinin signaling in cellular physiology of non-cardiac tissues

CCK signaling has been reported to have diverse roles in non-cardiac tissues, regulating cellular processes such as cell contraction, differentiation, and migration. These have been revealed using exogenous treatment with CCK peptide and the genetic deletion of Cck as well as Ccka/Cckb receptors. Unsurprisingly, many genetic studies of the role of CCK signaling have focused on phenotypes related to digestion and feeding behavior, and so far no studies have been reported which describe effects of CCK in cardiovascular disease models. Genetic deletion of the CCK gene resulted in mice which were viable, though increased somatostatin concentrations suggested compensatory mechanisms exist upon loss of CCK (Lacourse et al., 1999). CCK (-/-) mice were also reported to have increased food intake and increased fatty acid oxidation (Lo et al., 2008). Similarly, deletion of the Ccka receptor resulted in mice which are viable and which have feeding defects (Kopin et al., 1999). Furthermore, deletion of the Cckb receptor resulted in viable mice, though they had altered gastric pH, altered plasma gastrin concentrations, and increased proliferation of cells in the stomach and intestine (Nagata et al., 1996; Jin et al., 2009; Langhans et al., 1997). Surprisingly, loss of the Cckb receptor had no effect on pancreatic secretion or pancreatic weight (Miyasaka et al., 1999). However, overexpression of the Cckb receptor led to altered pancreatic acinar cell morphology and differentiation (Bierkamp et al., 2004). Double knockout mice for both Ccka and Cckb receptors were viable and displayed both greater energy expenditure and food intake, the same phenotype as for loss of the Cckb receptor alone (Miyasaka et al., 2002). Additionally, these mice showed increased gastric emptying (Miyasaka et al., 2004). Thus, genetic perturbation of CCK signaling components results in non-lethal phenotypes affecting the digestive system. However, the cardiovascular system of these mutant mice has not been studied.

In addition to the digestive system, CCK signaling has been implicated in neuronal cell differentiation and migration. For instance, exogenous CCK inhibited the migration of gonadotropin-releasing hormone-1 neurons of the olfactory bulb (Giacobini et al., 2004). Additionally, CCK (-/-) mice were

reported to have improved memory but also increased anxiety compared to wild type mice (Lo et al., 2008). However, knockout mice for the Cckb receptor displayed less anxiety during behavioral studies (Horinouchi et al., 2004). Furthermore, mutations in CCK, CCKAR, and CCKBR have been found which increase and decrease the likelihood of panic disorder (Koefoed et al., 2010). Additionally, a separate study identified mutations in the promoter of CCK which were associated with post-traumatic stress disorder (Badour et al., 2015).

Analysis of mutant embryos indicates that differences in adult mice might be due to subtle, non-lethal developmental perturbation. Ccka receptor and Cckb receptor mRNA is developmentally regulated in the brain, and Cckar (-/-)/Cckbr (-/-) double mutants displayed defects in neuronal differentiation and migration which led to mis-development of the cerebral cortex (Nishimura et al., 2015). In the forebrain of Ccka receptor (-/-) embryos, the number of GnRH neurons was greater compared to controls, suggesting that CCK inhibits GnRH neuronal differentiation (Giacobini et al., 2004). The CCK peptide has also been shown to be immunomodulatory, and this could potentially be through the regulation of the differentiation of T cells (Carrasco et al., 1997; De la Fuente et al., 1998; Meng et al., 2002; Zhang et al., 2014). Similar to ANP, CCK has also been reported to have effects on natriuresis. The Cckb receptor is expressed in the kidneys, including the proximal tubule and distal collecting ducts (de Weerth et al., 1998). Gastrin, which binds the Cckb receptor, caused a decrease in sodium reabsorption (von Schrenck et al., 2000). Thus, genetic studies have indicated that CCK signaling exerts effects on a variety of tissue and cell types, though the cardiovascular systems of these mutants have not yet been explored. These studies have led to its consideration as a ubiquitous messenger protein with endocrine, paracrine, and autocrine roles (Rehfeld, 2017).

2.5.4 Transcriptional regulation of proCck expression

Despite its significance in multiple organ systems, surprisingly little work has been performed on transcriptional regulation of proCck. In an intestinal cell line, protein hydrosylates were shown to increase proCck transcription, in line with the role of proCCK as a digestive peptide responding to nutritional intake (Cordier-Bussat et al., 1997). A separate study described a Cck-lacZ knock-in reporter mouse with embryonic expression in the nervous system and intestine (Lay et al., 1999). Images from the article also show expression in the heart (though not mentioned by the authors), but there was no further investigation of endogenous, cardiac proCck mRNA expression (Lay et al., 1999). Additionally, there are no reports of TFs underlying tissue-specific gene expression of cholecystokinin. In PC12 cells, a 100bp human proCCK promoter sequence was shown to be induced by KCl and forskolin, indicating that proCCK transcription was responsive to increases in intracellular levels of Ca^{2+} and cyclic adenosine monophosphate (cAMP), purportedly via protein kinase A (PKA) and extracellular signal-regulated kinase (ERK) signaling pathways (Hansen et al., 2004). Thus, to date there is no knowledge of tissue-specific regulators of proCck expression.

2.5.5 CCK signaling at a glance: the role of intracellular Ca^{2+}

Despite the known *in vivo*, physiological effects of exogenous CCK-8 on heart cell function, little to no work has been done on downstream effects of CCK signaling in cardiovascular cells. However, some work has been performed in other organ systems which can be used to infer potential effects of CCK signaling in the developing and adult heart. In murine pancreatic acinar cells, exogenous CCK-8 and CCK-58 treatment resulted in increases in intracellular Ca^{2+}, in addition to increased exocytosis of digestive enzymes (Criddle et al., 2009; Yule et al., 1991). Similar results were observed in isolated human pancreatic acinar cells, and these results were blocked by caffeine, suggesting the involvement of IP_3 channels in the mediation of CCK action (Murphy et al., 2008). Interestingly, the CCK activator

cerulean was shown to upregulate the transcription of Ca^{2+}-handling proteins Inositol 1,4,5-triphosphate receptor 1 (IP_3R1) and RyR2 genes in pancreatic acinar cells (Kim et al., 2019). Importantly, CCK was also shown to increase store-operated Ca^{2+} entry into dental enamel cells (Nurbaeva et al., 2018). In a separate study within murine acinar cells, CCK signaling was shown to promote the translocation of NFAT transcription factors into the nucleus, leading to the activation of NFAT-dependent promoters *in vitro* and *in vivo* (Gurda et al., 2008). This might be highly relevant to pathological hypertrophy models in the heart due to the importance of NFAT transcription factors during heart failure. Furthermore, exogenous CCK-8 activated MAPKs in primary sensory neurons isolated from mice, and these also play a role in the diseased heart (Yu et al., 2019). It is unknown whether CCK similarly regulates Ca^{2+} entry into cardiomyocytes. However, due to the aforementioned roles of intracellular Ca^{2+}(and MAP kinases) in heart failure, these findings indicate a potential mechanism for CCK-mediated modulation of cardiovascular function. To date, there are no reports of *in vivo* experiments involving genetic deletion or pharmacological perturbation of CCK signaling in heart failure models.

2.6 Chemical modulation of cardiac gene regulatory networks in congenital and adult cardiac diseases

Due to the wide variety of molecular entities involved in cardiogenesis, it is unsurprising that there are numerous druggable targets which can be perturbed chemically and lead to the disruption of this process *in utero*. The identification of such compounds has profound implications for fetal safety and the avoidance of the disastrous consequences which can result from the unnecessary creation of a new generation of congenital heart disease patients. On the other hand, identification of compounds capable of stimulating or inhibiting developmental processes might lead to innovative treatments for patients suffering from heart failure (generally well beyond their childbearing years). To this end, this section explores chemical compounds affecting cardiac developmental processes, methods by which they may be identified *in vitro*, and their potential for therapeutic uses in regenerative contexts. Importantly, this section builds upon the previous exploration of upstream signaling pathways, transcription factors, and cardiomyocyte subtype identity genes, which themselves may be modulated by chemical compounds (Figure 7).

2.6.1 Congenital heart diseases as a function of teratogenic compounds

Non-genetic sources of congenital heart malformation include maternal infections, chronic maternal illnesses, maternal exposure to teratogenic therapeutic and recreational drugs, and maternal exposure to teratogenic environmental toxins (Jenkins et al., 2007). Of 154 pregnancies examined in which fetuses were exposed to isotretinoin, a retinoid treatment for acne, there were 12 spontaneous abortions and 21 malformed infants (Lammer et al., 1985). Strikingly, the use of thalidomide to treat morning sickness in pregnant women led to the birth of over 10,000 children with congenital malformations (Vargesson, 2015). These are not isolated cases, as estimates suggest that teratogenic exposure (environmental or drug) may account for as many as 30% of CHD cases (Wilson et al., 1998). Molecular mechanisms of teratogenesis are believed to include folate antagonism, neural crest (progenitor) cell disruption, endocrine disruption, oxidative stress, vascular disruption, and specific-receptor or enzyme-mediated teratogenesis (van Gelder et al., 2010). Interestingly, there are similarities between Holt-Oram syndrome, the heart-hand syndrome caused by Tbx5 mutations, and certain manifestations of thalidomide embryopathy (Vargesson, 2015). Indeed, Tbx5 has been indicated as a potential direct target of thalidomide, or as one affected by thalidomide targeting of the

ubiquitination complex (Khalil et al., 2017; Vargesson, 2015). Thus, the analysis of phenocopies of genetic disease occurring due to teratogenic exposure can give insight into pathological mechanisms.

2.6.2 Stem cell-based *in vitro* modelling of teratogen-induced congenital heart malformation

Developmental and reproductive toxicity (DART) testing was developed in order to predict teratogenicity of drugs and industrial chemicals (Beekhuijzen, 2017). Unfortunately, these *in vivo* studies are cumbersome and require large numbers of animals (Beekhuijzen, 2017). It would thus be desirable to have high-throughput, predictive *in vitro* systems for the screening of novel drugs and industrial chemicals to determine teratogenic effects. To this end, the differentiation of embryonic stem cells to cardiomyocytes has been used to determine the embryotoxicity of novel drugs and environmental toxins through the validated embryonic stem cell test (EST) (Scholz et al., 1999; Seiler & Spielmann, 2011). Though these assays are not high-throughput, they do allow for *in vitro* testing of compounds for teratogenesis studies, and thus might allow for the avoidance of costly animal models. Importantly, the use of reporter lines might allow for improvement in both throughput and sensitivity of these methods. Though it is perhaps unlikely that these methods will replace DART in the near future, they could serve as a prescreening method before animal testing or as a method for the identification of teratogenic mechanisms of action.

2.6.3 Non stem cell-based *in vitro* and *in silico* models of cardiac gene transcription

Cell-based assays allow for rapid and efficient exploration of cardiac gene regulatory networks. Serial passaging of isolated rat ventricular tissue led to the isolation of H9c2 cells, immortal undifferentiated cardiomyoblasts which express sarcomeric and Ca^{2+} handling proteins in response to retinoid treatment, but do not beat spontaneously (Branco et al., 2015; Kimes & Brandt, 1976; Ménard et al., 1999). These cells can be used as a model of cardiac gene regulation. The rise in computational statistics might allow for the generation of in silico models which reveal aspects of cardiac gene regulatory networks. Interestingly, a thorough review of knockout phenotypes coupled with Boolean models of gene regulation were used to generate *in silico* models of developmental cardiac gene regulatory networks which could recapitulate expected phenotypes (Herrmann et al., 2012). Though not covered here in detail, additional cell-based and *in silico* models might allow for investigation of chemical modulation of cardiac gene regulatory networks.

2.6.4 Stem cell-based models of adult cardiac diseases for chemical screening

It is unclear whether stem cell-derived cardiomyocytes are mature enough to provide a faithful representation of adult human disease. For maturation into adult-like cardiomyocytes, it was reported that PSC-derived cardiomyocytes must be transplanted into the neonatal mouse heart, thus limiting the range of *in vitro* modelling to phenotypes which can be observed in embryonic cardiomyocytes (Cho et al., 2017). However, success has been reported in the modelling of adult diseases *in vitro* without transplantation to mice, despite the apparent embryonic phenotypes. For instance, retinoic-acid driven protocols directing pluripotent stem cells to form atrial cardiomyocytes were used to model atrial fibrillation through the analysis of characteristic arrhythmogenic rotors in cell sheets (Laksman et al., 2017). Additionally, three-dimensional engineered heart tissue derived from primary rodent and hIPSC-derived cardiomyocytes has been reported, including the generation of both artificial atria and ventricles used for drug screening (Breckwoldt et al., 2017; Krause et al., 2018; Lemme et al., 2018; Lemoine et al., 2017; MacQueen et al., 2018; Mannhardt et al., 2020). Refinement of the cardiomyocyte subtype specification protocols used in these models could lead to the development of cost-effective HTS systems for utilization in the pharmaceutical industry and

basic research. Thus, it is important to direct the cardiomyocyte subtype specificity of cardiomyocytes in order to development representative *in vitro* models. Though not mentioned here, there are myriad examples of the use of stem cell-derived cardiomyocytes to model cardiovascular diseases, using the existing models which have not been fully optimized in terms of subtype specificity and maturity of cardiomyocytes.

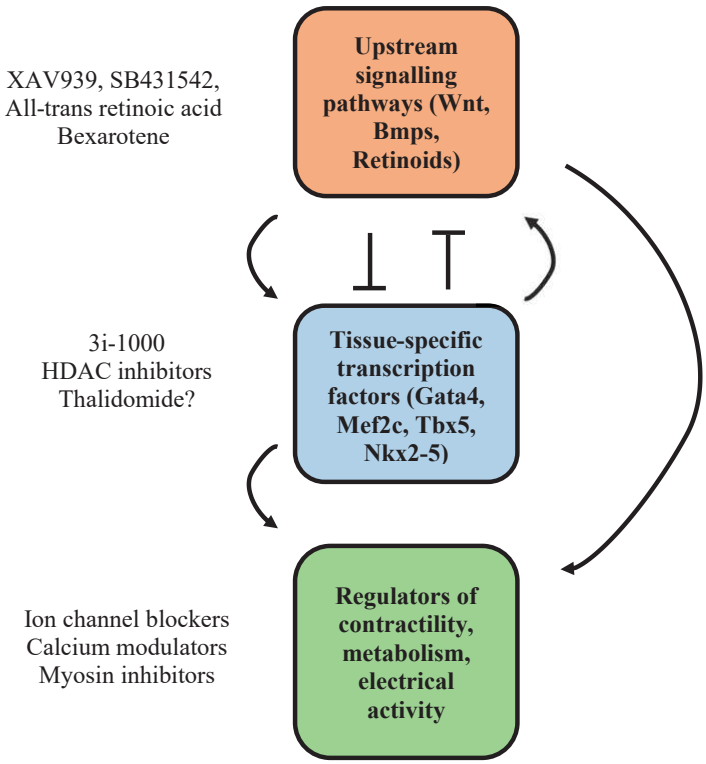

Figure 7. Levels of chemical modulation of cardiomyocyte differentiation and function. Known chemicals which target upstream signalling pathways, downstream transcription factors, and machinery governing contractile function. Within this framework, broad phenotypical changes could be induced by modulating both upstream signalling pathways and transcription factors, though these might result in more unpredictable outcomes than targeting specific members of the contractile machinery. Bone morphogenetic protein (Bmp), Histone deacetylase (HDAC), GATA transcription factor 4 (Gata4), Myocyte enhancer factor 2c (Mef2c), T-box transcription factor 5 (Tbx5), NK2 homeobox 5 (Nkx2-5).

2.6.5 Chemical modulation of cardiomyocyte differentiation of pluripotent stem cells

The rise of pluripotent stem cell technology has led to the identification of an increasing number of chemical compounds which promote or inhibit the differentiation of cardiomyocytes. A summary of these compounds is shown in Table 6. Several of these compounds also displayed teratogenic effects *in utero*. Unsurprisingly, many compounds affecting cardiomyocyte differentiation act via direct modulation of developmental signaling pathways discussed in previous sections, such as Wnt, BMP, and TGFβ. For instance, inhibition of BMP signaling with dorsomorphin enhanced cardiomyocyte differentiation when administered at early stages (Hao et al., 2008). In a separate study, high-content screening in mESCs led to the identification of ITD-1, a small molecule which promotes the degradation of Transforming Growth Factor Beta Receptor 2 (TGFBR2) (Willems et al., 2012). Interestingly, this compound increased cardiomyogenesis only when added after the mesodermal stage (Willems et al., 2012). Furthermore, the Wnt inhibitor XAV939 was shown to promote

cardiomyogenesis of mESCs (Wang et al., 2011). Shortly thereafter, it was discovered that chemical activation and subsequent inhibition of canonical Wnt signaling was sufficient to generate functional ventricular cardiomyocytes from human PSCs (Lian et al., 2012). Finally, chemical inhibition of p38 MAPK with SB203580 enhanced cardiomyocyte differentiation and activated c-Jun N-terminal kinase (JNK) signaling (Graichen et al., 2008). Thus, stage-specific inhibition or activation of developmental signaling pathways is a viable strategy for chemical differentiation of PSCs to the cardiomyocyte fate. In accordance with its embryonic function during cardiogenesis, studies on the effects of retinoic acid on the differentiation of PSCs have also been conducted. Addition of retinoic acid to late stages of mESC differentiation led to increases in sarcomeric gene expression, including activation of the ventricular-specific gene Myl2, providing early evidence that it promotes the differentiation of PSCs to cardiomyocytes (Wobus et al., 1997). Later studies also implicated positive effects of retinoic acid on atrial differentiation, specifically (Gassanov et al., 2008; Zhang et al., 2011). In a study which nicely recapitulated previous embryonic findings implicating both retinoic acid and BMP signaling in atrial and ventricular specification, the use of a fluorescent assay for aldehyde dehydrogenase allowed the generation of cardiogenic mesoderm which gave rise to the posterior (atrial) lineage by manipulation of BMP4 and Activin A concentrations (Lee et al., 2017). This study indicated that temporal differences in effects of retinoic acid on cardiomyocyte differentiation might be indicative of the metabolic machinery of the progenitor populations being measured.

There are also a few reports of the effects of epigenetic modulators on cardiomyocyte differentiation. In two separate studies, the DNA demethylating agent 5-azacytidine was shown to increase the differentiation of PSCs to the cardiomyocyte fate (Abbey & Seshagiri, 2013; Yoon et al., 2006). In a high-throughput screening assay, ascorbic acid was similarly shown to increase the differentiation of pluripotent stem cells to cardiomyocytes (Takahashi et al., 2003). A later study showed that this is related to demethylation effects of ascorbic acid, and this has been reported to enhance the catalytic activity of Ten-Eleven translocation family (TET) proteins, leading to global demethylation in mESCs (Abbey & Seshagiri, 2017; Yin et al., 2013). These studies suggest that DNA methylation maintains mESCs in an undifferentiated state, and removal of DNA methylation can be induced by chemical compounds in order to promote their differentiation.

In addition to compounds modulating developmental signaling pathways and epigenetic regulators, several cardiogenic compounds have been identified with as-yet-unknown mechanisms of action. A subset of (thio)urea and cinchona alkaloids were shown to enhance the cardiomyocyte differentiation of mESCs, though the molecular targets were not identified (Berkessel et al., 2010). Additionally, phenotypic screening for the induction of an Nppa-luciferase construct led to the identification of Cardiogenol C as a promoter of cardiomyogenesis (Wu et al., 2004). In a separate screen, sulfonyl-hydrazone (Shz) family molecules increased cardiomyocyte differentiation (Sadek et al., 2008). Additionally, ligands for peroxisome proliferator-activated receptors (PPARs) were modified, and structural derivatives increased the differentiation of mouse embryonic stem cells to cardiomyocytes as detected by an MHC-GFP reporter line (Wei et al., 2004).

Finally, some compounds have been identified which have atypical mechanisms of action. Verapamil, an L-type Ca^{2+} channel blocker, and cyclosporine, a protein phosphatase 2B inhibitor, increased the differentiation of pluripotent stem cells to cardiomyocytes (Sachinidis et al., 2006). Though originally reported to promote the differentiation of nodal cardiomyocytes, the potassium channel modulator EBIO was later shown to increase the proportion of cardiac progenitors by selective cell death of other progenitors (Jara-Avaca et al., 2017; Kleger et al., 2010). Collectively, these studies

compound	molecular (off) target (s) & pathway	effects on cardiogenesis	*in utero* effects	postnatal effects	citations	Applications
5-azacytidine	Cytidine analog, DNA demethylation	induces cardiomyocyte differentiation of PSCs	teratogenic	Cardioprotective post-MI, antifibrotic	Abbey & Seshagiri, 2013; Choi et al., 2004; Kim et al., 2014; Rosen et al., 1990; Yang et al., 2019; Yoon et al., 2006	in clinical use/investigation
Ascorbic acid	Tet/Free radicals, DNA demethylation/Anti oxidant	induces cardiomyocyte differentiation of PSCs	teratogenic at very high doses (6.68g / kg)	conflicting studies, clinical trials ongoing	Abbey & Seshagiri, 2017; Blaschke et al., 2013; Pillans et al., 1990; Rössig et al., 2001; Takahashi et al., 2003; Yin et al., 2013	in clinical use/investigation
all-trans retinoic acid	RAR/RXR, Retinoic acid	restricts growth of PSCs/mesodermal progenitors, stage-specific promotion of general cardiomyocyte fate/atrial cardiomyocytes, promotes atrial differentiation.	teratogenic	may attenuate remodelling in MI/aortic banding/angiotensin II models, but induce hypertrophy in normal conditions; may protect against arrhythmias.	Choudary et al., 2008; Gassanov et al., 2008; Kang & Leaf, 1995; Lee et al., 2017; Lü et al., 2003; Paiva et al., 2005; Silva et al., 2017; Wobus et al., 1997; Wang et al., 2002; Zhang et al., 2011	in clinical use/investigation
Cardiogenol	-	promotes cardiomyocyte differentiation of PSCs.	-	-	Wu et al., 2004	molecular probe

Table 6 (continued on three pages). Known chemical modulators of cardiomyocyte differentiation and cardiogenesis and their effects on adult cardiovascular function (if known). Most known chemical modulators of cardiomyocyte differentiation affect signalling pathways known via functional genetics studies to control cardiogenesis. Pluripotent stem cell (PSC), Myocardial infarction (MI), Ten-Eleven translocation family protein (Tet), Retinoic acid receptor (Rar), Retinoid x receptor (Rxr), Glycogen synthase kinase 3 alpha (GSK3α), Glycogen synthase kinase 3 beta (GSK3β), Second heart field (SHF), Nuclear factor of activated T cells (NFAT), Transcription factor (TF), Activin A Receptor Type 1 (Alk2), Bone Morphogenetic Protein Receptor Type 1A (Alk3), Bone Morphogenetic Protein Receptor Type 1B (Alk6), Bone morphogenetic protein (BMP), Isoxazole (Isx), NK2 homeobox 5 (Nkx2-5), Transforming Growth Factor Beta Receptor 2 (TGFBR2), Transforming growth factor β (TGF-β), T-box transcription factor (TBX5), Heart and neural crest derivatives expressed (HAND2), Mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), Activin A Receptor Type 1B (Alk4), Transforming Growth Factor Beta Receptor 1 (Alk5), Activin A Receptor Type 1C (Alk7), Histone deacetylase (HDAC).

compound	molecular (off) target (s) & pathway	effects on cardiogenesis	*in utero* effects	postnatal effects	citations	Applications
CHIR99021	GSK3α/GSK3β, Wnt (activator)	promotes cardiomyocyte differentiation of PSCs when added prior to mesoderm formation.	-	increases expression of Nav1.5 in adult cardiomyocytes *in vitro*.	Bennett et al., 2002; Lian et al., 2012; Martin et al., 2019	molecular probe
Cyclopamine	Smoothened, Hedgehog	impedes profliferation of SHF mesodermal progenitors	teratogenic	decreases Vegf mRNA in neonatal cardiomyocytes *in vitro*	Chen et al., 2002; Dyer & Kirby, 2009; Keeler, 1969; Lavine et al., 2008; Thomas et al., 2008	molecular probe
Cyclosporin	Protein phosphatase 2B, Calcineurin-Phosphatase Pathway/NFAT	Promotes cardiomyocyte differentiation of PSCs	not teratogenic	-	Sachinidis et al., 2006	in clinical use/investigation
DAPT	γ-secretase, Notch (inhibitor)	Promotes cardiomyocyte differentiation of PSCs	-	promotes direct cardiac reprogramming with cardiac TFs	Abad et al., 2017; Liu et al., 2014	molecular probe
Diclofenac sodium		-	potentially teratogenic	promotes direct cardiac reprogramming with cardiac TFs	Chan et al., 2001; Muraoka et al., 2019	in clinical use/investigation
Dorsomorphin	Alk2, Alk3, Alk6, BMP signaling	promotes cardiomyocyte differentiation of PSCs when added prior to mesoderm formation.	teratogenic	-	Hao et al., 2008; Yu et al., 2008	molecular probe
EBIO	SKCas, Ca2+-activated K+ signaling	promotes cardiomyocyte differentiation via selective cell death of non-cardiac progenitors, promotes pacemaker cell differentiation.	-	-	Jara-Avaca et al., 2017; Kleger et al., 2010	molecular probe

compound	molecular (off) target (s) & pathway	effects on cardiogenesis	*in utero* effects	postnatal effects	citations	Applications
ICG-001	ICG-001, Wnt (inhibitor)	unknown	-	improved ejection fraction, non-significant decrease in infarct size.	Sasaki et al., 2013	molecular probe
Isx	unknown, Nkx2-5/cardiac gene activator	promotes cardiomyocyte differentiation of PSCs	-	no functional improvement following MI, altered gene expression changes.	Russell et al., 2012; Sadek et al., 2008	molecular probe
ITD-1	TGFBR2 degradation, TGF-β (inhibitor)	Improves cardiomyocyte differentiation of PSCs if added after mesodermal stage	-	-	Schade et al., 2012; Willems et al., 2012	molecular probe
Lithium	GSK3β, Wnt (activator)	known to cause congenital heart defects	highly teratogenic	arrythmias and conduction disturbances exist as an adverse effect in humans.	Brady & Hogan, 1988; Klein & Melton, 1996	in clinical use/investigation
Pyrvinium	Casein Kinase 1α, Wnt (inhibitor)	unknown	-	conflicting studies *in vivo*, potentially beneficial effects on remodelling/fibrosis.	Saraswati et al., 2010; Thorne et al., 2010; Murakoshi et al., 2013	in clinical use/investigation
Thalidomide	Cerblon, TF degradation, TBX5/HAND2 interaction?, protein degradation, protein-protein interaction?	inhibits cardiomyocyte differentiation of PSCs	highly teratogenic, phenocopy of Holt-Oram syndrome	Potentially beneficial in chronic heart failure, reported to induce bradycardia and atrioventricular block as adverse effects during treatment for other disorders.	Donovan et al., 2018; Hinterseer et al., 2006; Khalil et al., 2017; Ma et al., 2015; Matyskiela et al., 2018; Vargesson, 2015; Yndestad et al., 2006	in clinical use/investigation

compound	molecular (off) target (s) & pathway	effects on cardiogenesis	*in utero* effects	postnatal effects	citations	Applications
SB203580	p38 MAPK, MAPK signaling, JNK signaling	improves cardiomyocyte differentiation of PSCs	-	enhanced contractility of adult rat cardiomyocytes via regulation of calcium handling proteins.	Graichen et al., 2008; Kaikkonen et al., 2014; Liao et al., 2002	molecular probe
SB431542	Alk4, Alk5, Alk7, TGF-β (inhibitor)	Improves cardiomyocyte differentiation of PSCs	-	promotes direct cardiac reprogramming with cardiac TFs *in vivo* post-MI, no functional change with only compound.	Drowley et al., 2016; Ifkovits et al., 2014; Mohamed et al., 2017	molecular probe
XAV939	Tankyrase 1 and Tankyrase 2, Wnt (inhibitor)	Improves cardiomyocyte differentiation of PSCs if added after mesodermal stage	-	promotes direct cardiac reprogramming with cardiac TFs *in vivo* post-MI, no functional change with only compound.	Huang et al., 2009; Mohamed et al., 2017; Wang et al., 2011	molecular probe
Valproic acid	HDACs; GSK3β?, Epigenetic signaling, Wnt signalling (activator)	inhibits cardiomyocyte differentiation of PSCs	teratogenic	Cardioprotective post-MI in rat model, potentially beneficial in humans.	Chen et al., 1999; Na et al., 2003; Olesen et al., 2011; Phiel et al., 2001; Tian et al., 2019; Wu et al., 2010	in clinical use/investigation

demonstrate that diverse molecular mechanisms promote the differentiation of cardiomyoyctes, including those to specific cardiomyocyte subtypes.

2.6.6 Chemical modulation of reprogramming of non-myocytes to the cardiomyocyte fate

In the past decade, reprogramming of non-cardiomyocytes to the cardiomyocyte fate has emerged as a strategy for cardiac regeneration. However, the generally low efficiency of reprogramming coupled with the need for gene transfer has led to an abundance of studies in which chemical compounds are used to enhance the reprogamming process or replace selected transcription factors. Interestingly, many of the compounds identified were previously known to promote the differentiation of cardiomyocytes from PSCs. For instance, treatment with the TGFβ inhibitor SB431542 resulted in a 5-fold increase in reprogramming efficiency driven by cardiac transcription factors GATA4, MEF2C,

TBX5, HAND2, and NKX2-5 (Ifkovits et al., 2014). A separate study confirmed the enhancement of reprogramming with the TGFβ inhibitor SB431542, in addition to showing improved reprogramming with the Wnt inhibitor XAV939 (Mohamed et al., 2017). DAPT, an inhibitor of the Notch pathway, markedly enhanced GMHT-mediated conversion of fibroblasts to induced cardiomyocytes, purportedly by increasing the binding of MEF2C to the promoters of sarcomeric genes (Abad et al., 2017). Screening of 8400 compounds revealed that diclofenac sodium, a non-steroidal anti-inflammatory drug, led to increased GMHT conversion of mouse and adult tail-tip fibroblasts (Muraoka et al., 2019). Astonishingly, a cocktail of nine chemical probes was used to convert human fibroblasts into spontaneously beating cardiomyocytes without the overexpression of any transcription factors or microRNAs (Cao et al., 2016). These results were only shown *in vitro*, and the combination of compounds used is highly unlikely to be feasible for clinical use. However, chemically-induced reprogramming and stem cell differentiation appear to share many overlapping mechanisms.

2.6.7 Chemical modulation of cardiomyocyte proliferation

Efforts have been undertaken to identify chemical inducers of cardiomyocyte proliferation, with the aim of regenerating infarcted myocardium. In a screen of a small chemical library (280 compounds) in mESC-derived cardiomyocytes, GSK-3 inhibitors (activators of Wnt pathway), p38 MAPK inhibitors, Ca(2+)/calmodulin-dependent protein kinase II inhibitors, and extracellular signal-related kinase activators increased cardiomyocyte proliferation (Uosaki et al., 2013). In a separate study, an overexpression screen of cell cycle regulators indicated that combinatorial overexpression of cyclin-dependent kinase 1, cyclin-dependent kinase 4, cyclin B1, and cyclin D1 were capable of inducing proliferation in post-mitotic cardiomyocytes, though chemical studies were not conducted (Mohamed et al., 2018). In large scale screening in the zebrafish model, the vitamin D analog alfacalcidol induced embryonic and adult cardiomyocyte proliferation (Han et al., 2019). However, this effect was not specific to cardiomyocytes and induced proliferation in a wide array of tissues (Han et al., 2019). Large-scale screening in neonatal cardiomyocytes showed that Carbacyclin, an activator of PPARδ and Wnt signaling, promoted the proliferation of postnatal cardiomyocytes (Magadum et al., 2017). Impressively, the small molecule PPARδ activator GW0742 induced proliferation in the post-MI heart and showed a reduction in infarct size and improved contractility (Magadum et al., 2017). Indeed, several compounds which affect differentiation and/or proliferation pathways of cardiomyocytes have shown beneficial effects *in vivo* (Table 6). Thus, developmental assays, such as differentiation or proliferation of embryonic cardiomyocytes might serve as powerful phenotypic screening tools for the identification of chemical targets relevant to *in vivo* function.

2.6.8 Molecular mechanisms of heart failure and current treatments

Heart failure is characterized by a decline in cardiac output triggered by myocardial infarction, viral myocarditis, or genetic factors (Kaye & Krum, 2007; Mazurek & Jessup, 2017). The initial damage to the heart by one of these causes leads to activation of the sympathetic nervous system, and this in turn causes increases in heart rate and stroke volume (Kaye & Krum, 2007; Mazurek & Jessup, 2017). Neurohormones activated by this process include the renin-angiotensin system, ANP/BNP, ET-1, and cytokines (Kaye & Krum, 2007; Mazurek & Jessup, 2017). The activation of these pathways thereby induces cardiomyocyte hypertrophy, fibrosis, and left-ventricular remodeling (Kaye & Krum, 2007). Treatment mainstays for this condition include diuretics, ACE inhibitors, angiotensin receptor blockers, angiotensin receptor/neprilysin inhibitors, mineralocorticoid receptor antagonists, and β-adrenoceptor antagonists (beta blockers), though the combinations of these can vary depending on

the stage of disease and symptoms (Kaye & Krum, 2007; Mazurek & Jessup, 2017). In clinical trials, infusion of ANP/BNP resulted in improved hemodynamic function, though larger studies found that BNP infusion does not result in decreases in death or re-hospitalization (Colucci et al., 2000; O'Connor et al., 2011; Yasue & Yoshimura, 1996). However, intramyocardial delivery of BNP resulted in improved myocardial function in rats, suggesting its local effects on cardiac cells might be further developed as a therapeutic avenue (Moilanen et al., 2011). Despite these treatment advances, the survival outlook for patients remains poor, leading researchers to search for new drug targets which can attenuate pathological remodeling. It is thus highly interesting to investigate downstream pathways and targets which could help ameliorate nefarious remodeling, including via direct or indirect modulation of developmental gene regulatory networks re-activated during pathological conditions.

2.6.9 Chemical inhibition of the pathological gene regulatory response- a new way forward?

Recent efforts have focused on new classes of molecular targets in heart failure and cardiovascular diseases, such as contractile proteins, cytoskeletal proteins, Ca^{2+}-handling proteins, G-protein coupled receptors, cell signaling molecules, epigenetic modifiers, transcription factors, peptide hormones/receptors, oxidases, and growth factors (Dhaun & Webb, 2019; Dridi et al., 2020; Kaye & Krum, 2007; Li et al., 2020; Lim, 2020; Teerlink et al., 2020; Wallner et al., 2020; Zhang et al., 2020). The selection of targets has often been driven by known genetic gain- or loss-of-function phenotypes, and targets regulating transcriptional networks have been actively explored. For instance, increased calcineurin/NFAT signaling resulted in re-expression of Hand2 during heart failure, leading to induction of developmental gene regulatory networks and pathological hypertrophy (Dirkx et al., 2013). Additionally, calcineurin-induced hypertrophy led to the protection of cardiomyocytes from apoptosis via an increase in NFAT activity (De Windt et al., 2000). Similarly, genetic disruption of p38 MAPK signaling resulted in increases in pathological hypertrophy, reportedly by increasing the calcineurin-NFAT transcriptional response (Braz et al., 2003). In this line, the calcineurin inhibitors cyclosporine and FK506 were shown to prevent heart failure in mouse models of both genetic and pressure-overload hypertrophy (Sussman et al., 1998). However, the clinical utility of these approaches is limited due to a lack of cardioselectivity, and therefore the potential for an increase in toxicity (Kaye & Krum, 2007).

Intriguingly, many compounds which affect differentiation pathways have also been shown to affect adult cardiovascular phenotypes, and a summary of relevant studies is shown in Table 6. In some cases, these developmentally active compounds have been shown to attenuate pathological remodeling of the heart. For instance, exogenous retinoids suppressed neurohormonal signaling (endothelin, Nppa) and cardiomyocyte hypertrophy in response to phenylephrine stimulation (Zhou et al., 1995). Similarly, *all-trans* retinoic acid was reported to impede pathological remodeling in rats subjected to aortic banding, and in another study was shown to reduce arrhythmias generated by disease stimuli (Choudhary et al., 2008; Kang & Leaf, 1995). In conjunction with overexpression of cardiac transcription factors, inhibitors of Wnt (XAV939) and Tgfβ (SB431542) signaling pathways promoted the direct reprogramming of fibroblasts to cardiomyocytes *in vivo*, improving cardiac function (Mohamed et al., 2017). Similarly, DAPT, a Notch inhibitor, both promoted the differentiation of cardiomyocytes and the *in vivo* reprogramming of fibroblasts to the cardiomyocyte fate (Abad et al., 2017; Liu et al., 2014).

Modulators of epigenetic pathways have also been explored as therapeutic agents *in vivo*. The differentiation inducer and DNA methylation modifier 5-azacytidine was reported to be

cardioprotective and antifibrotic post-MI (Kim et al., 2014). Another differentiation modulator and teratogen, valproic acid, was reported to be cardioprotective in rats post-MI, obstensibly via inhibition of HDACs (Kee et al., 2006; Tian et al., 2019). In patients treated for epilepsy, valproic acid was also reported to reduce the risk of myocardial infarction (Olesen et al., 2011). Even the teratogen thalidomide has been shown to be beneficial in post-infarction remodeling in rats, possibly due to its anti-fibrotic effects (Yndestad et al., 2006). Some compounds affecting cardiomyocyte differentiation/cardiogenesis produce adverse cardiac effects when given to patients. For instance, the teratogen and TBX5 modulator thalidomide has been reported to induce conduction system dysfunction (Hinterseer et al., 2006). Furthermore, lithium, a teratogen and GSK3β inhibitor (Wnt pathway activator), is also known to induce arrhythmias and conduction system abnormalities (Brady & Hogan, 1988). Collectively, these studies indicate the potential utility of chemical modulation of developmental pathways in the diseased adult heart. Additionally, they suggest that phenotypic screening using stem cell-based models could lead to the identification of bioactive compounds relevant to the treatment of adult disease or the identification of off-target cardiovascular effects.

3 Aims of the studies

I. Develop and validate cell-based models for the specification of atrial and ventricular cardiomyocytes.

II. Perform chemical screening to identify the role of GATA4-targeted compounds on the differentiation of cardiomyocytes from pluripotent stem cells. Analyze biological effects of hit compounds and investigate mechanism-of-action.

III. Characterize the developmental expression of procholecystokinin in the heart and investigate its regulation at the transcriptional level by the chamber-specific transcription factor Tbx5. Explore the effects of exogenous CCK octapeptide on the differentiation of cardiomyocytes.

4 Materials and methods

A detailed description of methods can be found in the component articles I-III. A summary of methods utilized is found in Table 7.

4.1 Characterization of tissue and cell culture samples by qRT-PCR (Studies I, II, & III)

In order to obtain embryonic samples for analysis, timed matings of C57BL/6JOlaHsd mice were performed. Embryos were dissected from the uteruses of pregnant mice and embryonic samples were collected into Trizol Reagent. Purity of these samples was enhanced using RNeasy MinElute Cleanup kit (Qiagen). Embryoid bodies (EBs) from spontaneous differentiation of mESCs were similarly processed. RNA from primary cardiomyocye cultures was isolated using the NucleoSpin® RNA isolation kit (Macherey-Nagel). Taqman gene expression assays were obtained from ThermoFisher Scientific and qRT-PCR reactions were performed on a Fluidigm Biomark HD system at the Functional Genomics Unit, University of Helsinki or on a Roche 480 Light Cycler. Values were normalized to a reference gene (Tbp for samples from myocardial infarction, Actb for other samples).

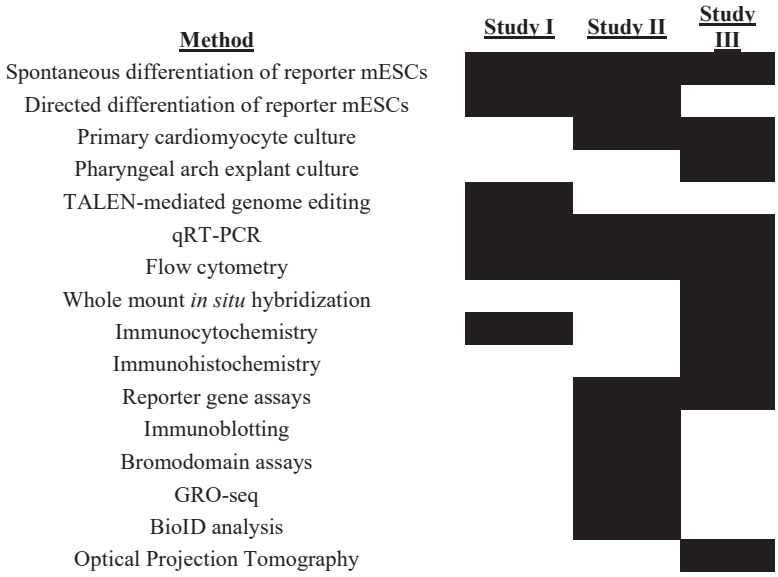

Table 7. Methods utilized in the studies composing the thesis. Transcription activator-like effector nuclease (TALEN), Quantitative real-time polymerase chain reaction (qRT-PCR), Global run-on sequencing (GRO-seq), proximity-dependent biotin identification (BioID).

4.2 Culture and differentiation of pluripotent stem cells (Studies I, II, & III)

E14 mouse embryonic stem cells were cultured in the presence of leukocyte inhibitory factor (LIF) on 0.1% Gelatin in order to maintain pluripotency. For spontaneous differentiation of mESCs, cells were allowed to form EBs in inverted v-bottomed plates without the presence of LIF. EBs were subsequently transferred to adherent plates and coated with 0.1% Gelatin to allow the formation of spontaneously beating cardiomyocytes. Compounds were added to differentiation cultures as indicated. Cardiomyocyte differentiation efficiency was assessed by flow cytometric analysis of reporter expression or qRT-PCR for cardiac genes. Directed differentiation of wild type and reporter mESCs was performed essentially as described (Kattman et al., 2011) but instead of plating to

adherent conditions at D4 (mesodermal) stage, cells were grown in suspension in ultra-low attachment plates in order to generate sufficient cell material for compound screening. Progenitor cells were plated to 384-well black plates, and venGFP fluorescence was assessed using a Pherastar fluorescent plate reader (BMG).

4.3 Genome editing of pluripotent stem cells to generate reporter cell lines for compound screening (Study I)

Transcription activator-like effector nuclease (TALEN)-mediated genome editing was utilized in order to modify mESCs to generate reporter cell lines. TALEN-encoding plasmids targeting the ventricle-specific Myl2 gene were synthesized according to published methods (Cermak et al., 2010). A donor plasmid containing an in-frame eGFP reporter cassette was synthesized using InFusion HD cloning. TALEN-encoding plasmids were co-transfected with donor plasmids into mESCs using Xfect transfection reagent (Clontech). Clones with targeted eGFP reporter cassette integration at the Myl2 locus were identified by Junction PCR. A transgene in which the atrial-specific quail SMyHC3 promoter drives the TdTomato fluorescent reporter was assembled using InFusion HD cloning. This construct was transfected into mESCs, and double positive clones were identified by PCR genotyping. Reporter mESCs were subsequently passaged and re-genotyped to confirm stable integration. Reporter expression was confirmed *in vivo* by laser-assisted morula injection and imaging of chimeric embryos (Poueymirou et al., 2007).

4.4 Flow cytometry (Studies I, II, & III)

EBs from differentiation experiments were dissociated with TrypLE. TrypLE was inactivated with serum, and cells were washed 1x with PBS prior to cell straining. Flow cytometry was performed on a BD LSRFortessa flow cytometer, and wild type and single reporter mESC-derived EBs were used as negative controls for gating. Analysis of flow cytometry data was performed using Flowjo (Flowjo LLC). Values were normalized to control and statistical analysis was performed in R.

4.5 Whole mount *in situ* hybridization (Study III)

Embryos were dissected from pregnant wild type mice (C57BL/6JOlaHsd). Embryos were fixed in 4% Paraformaldehyde (PFA) and stained as described. In situ probes were amplified from embryonic heart cDNA using the following primers and labelled with digoxigenin-11-UTP.

proCck:	F: 5’-CCTCAACTTAGCTGGACTGCAG-3’
	R: 5’-CGGTCACTTATTCTATGGCTGG-3’
Cckar:	F: 5’-GCTGCCACCTGGTGCCTCTC-3’
	R: 5’-CCGCCAGGCATTGGCACTGA-3’
Cckbr:	F: 5’-GCCGGGTCCGAAACCAAGGG-3’
	R: 5’-TCCCTCAGCCAGGTCCCAGC-3’

Whole mount in situ hybridization (ISH) using digoxigenin-conjugated probes and BMpurple was performed as previously described (Correia & Conlon, 2001). Staining of neonatal hearts required an extension of permeabilization, pre-hybridization, and washing steps. Embryos and/or neonatal hearts were imaged on a stereomicroscope (Olympus) or by Optical projection tomography. Samples for optical projection tomography were prepared essentially as described (Sharpe, 2003). Optical

projection tomography was performed using a Bioptonics 3001 device, and images were reconstructed and analyzed using NRECON and AMIRA 5.0 (ThermoFisher), respectively.

4.6 Immunocytochemistry (Study I & III)

Cell cultures were washed with PBS and fixed for 10 minutes in 4% PFA. Cells were permeabilized with 0.5% Triton-X and stained overnight with primary antibodies at 4 degrees. The following day, cells were washed repeatedly (1 hour) and stained with a fluorescent-conjugated secondary antibody. For study III, PA1 and PA2 were cultured as described (Andersen & Kwon, 2015). Primary antibodies used for characterization of mESCs and mESC-derived cardiomyocytes include those raised against Oct4 (sc-8628), MF20 (Developmental Studies Hybridoma Bank), α-actinin (Sigma-Aldrich), Isl1 (Developmental Studies Hybridoma Bank), RFP (Rockland), and cardiac troponin T (Thermo Fisher Scientific). Secondary antibodies were Alexa-Fluor conjugated (ThermoFisher Scientific), and DAPI was used as a nuclear counterstain. Stained samples were imaged on a Leica DMi8 inverted fluorescent microscope.

4.7 Immunohistochemistry (Study III)

proCCK peptide was detected in 15μM cryosections using a commercial primary antibody (Cloud-Clone Corporation, PAA802Mu01) and a goat anti-rabbit HRP-conjugated secondary antibody (Jackson) following the manufacturer's instructions. A Pannoramic Digital Slide Scanner (3DHISTECH) was used to image stained tissue sections.

4.8 DNA motif analysis (Study III)

DNA motif analysis was conducted on the 10000bp region flanking the proCck transcriptional start site in R using Motif Counter (Kopp & Vingron, 2017). Position frequency matrices representing transcription factor motifs were obtained from MotifDb (Bioconductor). Tbx5 motifs were also extracted from a previously published study (Luna-Zurita et al., 2016). Analysis was first conducted in the full 10000bp region to identify putative regulators. For Tbx5 and Mef2c, the 10kb region was broken into 500bp fragments in order to identify regions with enriched Tbx5 and Mef2c binding regions. To identify enriched motifs in the proCck regulatory region, the beta-actin promoter was used as a background sequence as it is ubiquitously expressed.

4.9 Reporter gene assays (Study II and III)

A putative regulatory region including 2500bp flanking the mouse proCck transcriptional start site was cloned upstream of the firefly luciferase gene in a pGL3-Basic vector by Gibson assembly. For overexpression of TFs, the plasmids FUdeltaGW-rtTA (Addgene #19780), tetO-TBX5 (Addgene #46032), tetO-MEF2C (Addgene #46031), and tetO-GATA4 (Addgene #46030) were utilized. A renilla vector lacking GATA4 binding sites (pRL-TK-d238) was used to control for cell number and transfection efficiency (Ho & Strauss, 2004). HEK293 cells were transfected with reporter and TF overexpression constructs using Lipofectamine 3000 (Thermo Fisher Scientific). Reporter assays were performed using a Dual Luciferase reporter kit (Promega) and the luciferase signal was measured using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific). Firefly/renilla luciferase were calculated and normalized to controls. For assessment of reporter activity in primary cardiomyocytes, neonatal rat cardiomyocytes were isolated as described (Karhu et al., 2018). proCck-luciferase and Nppa-540-luciferase constructs were delivered using Lipofectamine 3000. Transfected cells were stimulated with ET-1 (Sigma-Aldrich) and Isoprenaline HCL (Sigma-Aldrich) to gauge response to pathological stimuli. Statistical analysis was conducted

in R. Reporter gene assays in Study II were performed in COS-1 cells similar to in a previous study (Kinnunen et al., 2018).

4.10 Immunoblotting (Study II)

A lentiviral-encoding plasmid for doxycycline-inducible overexpression of a Gata4-V5 cassette was obtained from Addgene (#46030), and viral production was performed at the Biomedicum Virus Core at the University of Helsinki. Primary rat cardiomyocytes were transduced with lentiviral particles, whereas HEK293 cells were transfected with lentiviral-encoding plasmids using Lipofectamine 3000. Cells were lysed in Laemmli buffer with 2-mercaptoethanol and boiled for 5 minutes. SDS-PAGE was conducted using antibodies for detection of GATA4 (Santa Cruz Biotechnology, sc-9053), V5 (Cell Signaling Technology, #13202), and β-actin (Cell Signaling Technology, #4967).

4.11 Compounds used for testing

9-cis retinoic acid: Sigma-Aldrich; All-trans retinoic acid: Sigma-Aldrich; Bexarotene: Sigma-Aldrich; SB431542: Sigma-Aldrich; XAV939: Sigma-Aldrich; JQ1: Sigma-Aldrich; GATA4-targeted compounds: Jumppanen et al., 2019. Compounds were diluted in DMSO prior to cell culture treatments.

4.12 Bromodomain assays (Study II)

Bromodomain assays were conducted at DiscoverX to identify interactions of compounds with 32 bromodomain-containing proteins according to established methods (Fabian et al., 2005).

Percent control was calculated as follows:

((test compound signal – positive control signal) / (negative control signal – positive control signal)) *100

4.13 GRO-seq analysis (Study II)

In order to identify gene transcription induced or inhibited by compound treatment, GRO-seq was performed in neonatal rat ventricular cardiomyoyctes. Neonatal cardiomyocytes were cultured as previously described (Välimäki et al., 2017), and GRO-seq experiments were performed as published (Kaikkonen et al., 2013).

4.14 BioID analysis (Study II)

The GATA4 and NKX2-5 interactomes were analyzed using the proximity-dependent biotin identification (BioID) method in HEK293 cells as described (Liu et al., 2018). For analysis, filtering was carried out using the CRAPome repository (Mellacheruvu et al., 2013).

5 Results

5.1 STUDY I

In study I, a reporter system was developed for the differentiation of PSCs to atrial and ventricular cardiomyocyte subtypes. Candidate marker genes were measured in embryonic day 10 (E10) mouse atria and ventricles by qRT-PCR in order to determine their specificity to embryonic cardiac chambers. Robust expression of Myl2 was detected in embryonic ventricles, but only low levels were detected within embryonic atria, suggesting its suitability as a ventricular marker gene. Based on this analysis, Myl2 was selected for genomic modification, and an eGFP reporter was integrated into the endogenous Myl2 locus by TALEN-mediated genome editing to generate a ventricular-specific allele (Myl2-eGFP; venGFP). Due to the lack of an endogenously expressed atrial marker, an atrial-specific transgene (SMyHC3-TdTomato; atrRFP) was integrated into the same reporter line to generate a dual reporter PSC line to measure differentiation to ventricular and atrial lineages simultaneously.

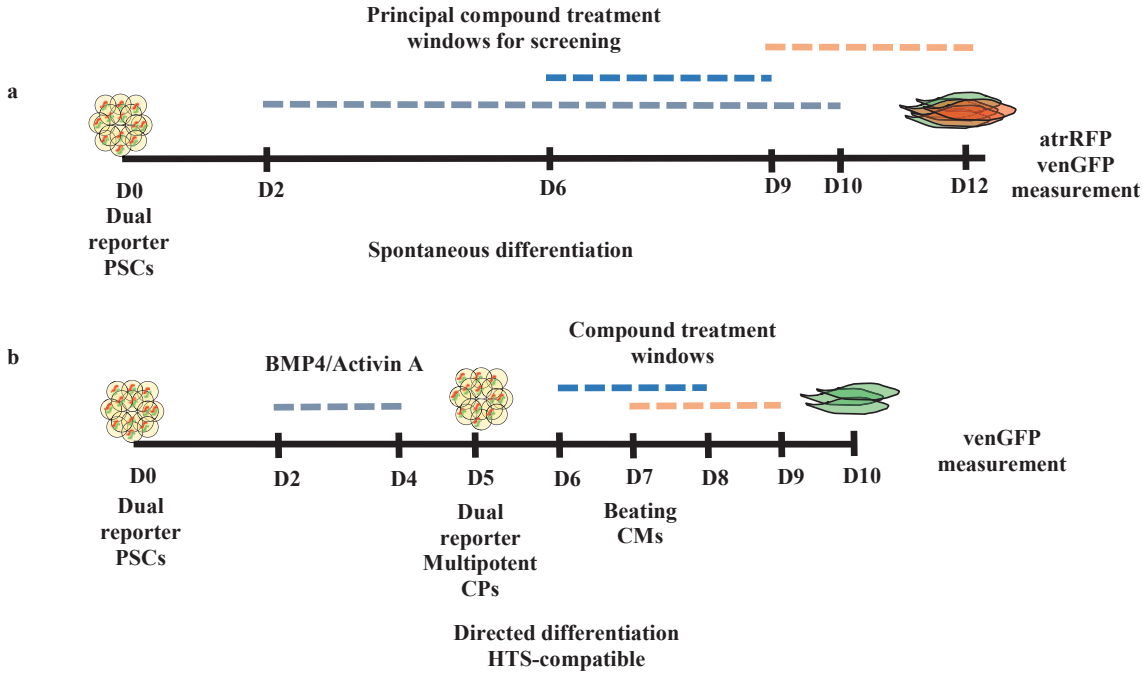

Figure 8. Cardiomyocyte differentiation protocols used in the current studies. **a** Spontaneous differentiation protocol used for compound/peptide screening in Studies I, II, & III. Cell differentiation conducted in the presence of serum and allowing for the measurement of both atrial and ventricular differentiation by flow cytometry. Shorter compound treatment windows were utilized in Study I encompassing time points before (D6-D9) and after (D9-D12) the onset of spontaneous beating to study the effects of retinoids. Study II utilized a longer treatment window (D2-D10) to study the effects of compounds that inhibit GATA4/NKX2-5 transcriptional synergy. **b** Directed differentiation protocol used for compound screening in Studies I & II. Cell differentiation in the absence of serum and the measurement of only ventricular differentiation medium by a plate reader system. PSCs were induced to become cardiac mesoderm by BMP4/Activin A, and multipotent cardiac progenitors were plated into 384-well plates for compound screening. Multipotent CPs were Isl1 expressing and thus predominantly represented cells of the second heart field. Pluripotent stem cell (PSC), Atrial-specific (posterior) fluorescent reporter (atrRFP), Ventricular-specific (anterior) fluorescent reporter (venGFP), Cardiac progenitor (CP), Cardiomyocyte (CM), High-Throughput Screening (HTS), GATA transcription factor 4 (GATA4), NK2 homeobox 5 (NKX2-5), Bone morphogenetic protein 4 (BMP4).

Dual reporter mESCs were subsequently used to make chimeric embryos, and reporter expression was observed in respective cardiac chambers of embryonic mice (Table 8). In order to determine the effects of chemical compounds on the differentiation process, spontaneous and directed differentiation assays were developed. In the spontaneous differentiation assay, compounds were added either before (D6-D9) or after (D9-D12) the onset of spontaneous beating (Figure 8a). Among

Gene	Stage	Expression
Myl2-eGFP/venGFP	E7.5	cardiac crescent
Myl2-eGFP/venGFP	E8.25	primitive ventricle
Myl2-eGFP/venGFP	E8.5	primitive ventricle
Myl2-eGFP/venGFP	E8.75	primitive ventricle
Myl2-eGFP/venGFP	E9.5	left ventricle, atrioventricular canal, right ventricle, outflow tract
SMyHC3-TdTomato/atrRFP	E7.5	not present
SMyHC3-TdTomato/atrRFP	E8.25	inflow region, sinus horns
SMyHC3-TdTomato/atrRFP	E8.5	primitive atria
SMyHC3-TdTomato/atrRFP	E8.75	primitive atria
SMyHC3-TdTomato/atrRFP	E9.5	left atria, atrioventricular canal, right atria
proCCK	E8.0	linear heart tube
proCCK	E8.5-E9.0	future interventricular septum, future left/right ventricles, base of the atria
proCCK	E9.5	base of atria, right/left ventricle
proCCK	E9.75	base of atria, right/left ventricle
proCCK	E10.5	base of atria, right/left ventricle, base of outflow tract
proCCK	P1 neonatal	left ventricle, right ventricle near the interventricular wall, right and left atria

Table 8. Expression of novel chamber-specific markers during mouse embryogenesis. Expression pattern of proCholecystokinin in the embryonic and neonatal heart as measured by whole mount in situ hybridization of wild type embryos. Expression of Myl2-eGFP and SMyHC3-TdT as measured by fluorescence microscopy of reporter embryos. Myosin light chain 2 (Myl2), Ventricular-specific (anterior) fluorescent reporter (venGFP), Slow myosin heavy chain 3 (SMyHC3), Atrial-specific (posterior) fluorescent reporter (atrRFP), procholecystokinin (proCCK).

the compounds tested were all-trans retinoic acid, 9-cis retinoic acid, and bexarotene. Parameters measured during these experiments included the %venGFP+ cells and %atrRFP+ cells, in addition to the mean fluorescent intensity (MFI) of venGFP and atrRFP. In order to assess subtype-specific effects of chemical modulators, the atrRFP/venGFP ratio was measured, both for the % cells and the MFI. A summary of differentiation results is shown in Table 9. Interestingly, increased sensitivity to retinoids was observed when added before the onset of spontaneous beating, indicating that compounds specifically affect undifferentiated cardiac progenitors. Furthermore, increased sensitivity was observed for dual RAR/RXR agonists (all-trans retinoic acid) than for RXR agonists (bexarotene). Importantly, 1μM all-trans retinoic acid displayed an atrializing effect on cardiac progenitors, as measured by %atrRFP/%venGFP. In addition to the spontaneous differentiation assay, a directed differentiation protocol was developed based on large-scale production of multipotent cardiac progenitors for compound screening in 384-well plates (Figure 8b). As in the spontaneous differentiation assay, compounds were added before (D6-D8) and after (D7-D9) the onset of beating. All-trans retinoic acid strongly inhibited differentiation when added at the cardiac progenitor (CP) stage, while promoting the expression of venGFP when added at the beating cardiomyocyte stage. In addition to retinoids, modulators of Wnt (XAV939) and TGFβ (SB431542) pathways promoted ventricular reporter expression when added to multipotent cardiac progenitors, but had no effect on reporter expression when added to differentiated cardiomyocytes.

5.2 STUDY II

In study II, the differentiation assay established during study I was used to screen chemical compounds that interfere with the transcriptional synergy of core cardiac transcription factors GATA4 and NKX2-5. Compounds were screened in a broad window representing mesodermal specification, cardiac progenitor commitment, and activation of cardiomyocyte differentiation programs. Primary screening led to the identification of compounds promoting the activation and repression of atrial and ventricular reporters, such as 3i-1000 and 3i-1103 (Results in Table 8). Importantly, these compounds increased the proportion of cells expressing atrRFP and venGFP markers.

Based on these results, compound addition windows were further modified based on the directed differentiation assay in study I, and it was shown that short compound addition (2-day) to multipotent cardiac progenitors led to activation of the ventricular-specific reporter gene. Additionally, immunoblotting revealed that 3i-1000 decreased levels of the well-known 50 kDa band of GATA4, while increasing levels of a previously undescribed 70 kDa band of GATA4. These results suggest that compounds exert effects on differentiation by modulating the molecular isoforms of the GATA4 transcription factor. Analysis of the structures of active compounds indicated the involvement of an acetyl-lysine like fragment that was present in active compounds. Furthermore, analysis of the GATA4 protein interactome by BioID indicated broad interactions between the GATA4 protein and bromodomain-containing proteins. Furthermore, 3i-1000 was revealed to attenuate activation of the chamber-specific Nppb promoter by the BET bromodomain inhibitor JQ1, supporting both the relationship between GATA4 and bromodomain-containing proteins and the acetyl-lysine like fragment present in 3i-1000. Finally, GRO-seq analysis in primary neonatal cardiomyocytes revealed regulation of genes related to both cell fate determination and regeneration upon treatment with 3i-1000.

Treatment	Dose (μM)	Treatment Window	%venGFP	venGFP MFI	%atrRFP	atrRFP MFI	%atrRFP/ %venGFP	atrRFP MFI/venGFP MFI	Study
all-trans retinoic acid	0.1	D6-D9	0.59 ± 0.16	0.80 ± 0.18	1.06 ± 0.19	**0.71 ± 0.11**	**2.01 ± 0.34**	1.16 ± 0.39	I
all-trans retinoic acid	1	D6-D9	**0.23 ± 0.09**	**0.47 ± 0.09**	**0.49 ± 0.11**	**0.47 ± 0.07**	3.24 ± 1.06	1.02 ± 0.06	I
all-trans retinoic acid	10	D6-D9	**0.09 ± 0.02**	**0.34 ± 0.06**	**0.16 ± 0.05**	**0.55 ± 0.07**	1.94 ± 0.55	**1.74 ± 0.26**	I
all-trans retinoic acid	0.1	D9-D12	0.82 ± 0.14	1.20 ± 0.15	1.23 ± 0.31	**0.78 ± 0.04**	1.90 ± 0.76	**0.67 ± 0.05**	I
all-trans retinoic acid	1	D9-D12	**0.55 ± 0.13**	1.33 ± 0.25	**0.43 ± 0.12**	**0.59 ± 0.09**	0.82 ± 0.14	**0.48 ± 0.08**	I
all-trans retinoic acid	10	D9-D12	**0.60 ± 0.09**	1.79 ± 0.46	0.63 ± 0.14	**0.52 ± 0.09**	1.18 ± 0.37	**0.39 ± 0.13**	I
9-cis retinoic acid	0.1	D6-D9	**0.40 ± 0.05**	0.87 ± 0.11	1.54 ± 0.80	0.77 ± 0.12	4.96 ± 3.33	0.99 ± 0.28	I
9-cis retinoic acid	1	D6-D9	**0.11 ± 0.01**	**0.39 ± 0.05**	**0.25 ± 0.07**	0.53 ± 0.07	2.23 ± 0.61	1.41 ± 0.26	I
9-cis retinoic acid	10	D6-D9	**0.29 ± 0.17**	**0.44 ± 0.10**	0.77 ± 0.56	0.45 ± 0.13	1.97 ± 0.65	1.13 ± 0.26	I
9-cis retinoic acid	0.1	D9-D12	0.79 ± 0.09	1.07 ± 0.08	2.18 ± 1.12	0.94 ± 0.15	2.81 ± 1.22	0.87 ± 0.12	I
9-cis retinoic acid	1	D9-D12	**0.60 ± 0.11**	1.63 ± 0.40	0.56 ± 0.17	0.78 ± 0.15	1.02 ± 0.27	0.62 ± 0.22	I
9-cis retinoic acid	10	D9-D12	**0.60 ± 0.14**	**1.76 ± 0.31**	1.45 ± 0.77	**0.59 ± 0.04**	2.25 ± 0.86	**0.38 ± 0.07**	I
Bexarotene	0.1	D6-D9	0.87 ± 0.08	0.83 ± 0.12	1.69 ± 0.64	0.94 ± 0.10	2.25 ± 1.08	1.17 ± 0.10	I

Table 9 (continued on next pages). Effects of retinoids, GATA4-targeted compounds, and CCK-8 peptide on the spontaneous differentiation of PSCs. Compilation of data from Studies I, II, and III displays the effects of diverse agents on the specification of atrial and ventricular cardiomyocytes. Values are normalized to DMSO control ± SEM. Ventricular-specific (anterior) fluorescent reporter (venGFP), Atrial-specific (posterior) fluorescent reporter (atrRFP), Mean fluorescent intensity (MFI). Statistically significant changes are underlined in **bold**.

Treatment	Dose (µM)	Treatment Window	%venGFP	venGFP MFI	%atrRFP	atrRFP MFI	%atrRFP/ %venGFP	atrRFP MFI/venGFP MFI	Study
Bexarotene	1	D6-D9	0.89 ± 0.07	**0.62 ± 0.08**	1.88 ± 0.49	0.96 ± 0.15	2.05 ± 0.51	1.67 ± 0.38	I
Bexarotene	10	D6-D9	0.58 ± 0.20	**0.54 ± 0.10**	0.85 ± 0.36	1.06 ± 0.14	2.49 ± 1.20	**2.22 ± 0.54**	I
Bexarotene	0.1	D9-D12	0.81 ± 0.09	1.02 ± 0.12	2.49 ± 1.17	1.02 ± 0.13	2.69 ± 0.95	1.10 ± 0.25	I
Bexarotene	1	D9-D12	1.07 ± 0.21	1.22 ± 0.18	1.27 ± 0.34	0.97 ± 0.09	1.13 ± 0.12	0.89 ± 0.19	I
Bexarotene	10	D9-D12	0.78 ± 0.20	**1.73 ± 0.30**	1.45 ± 0.31	1.15 ± 0.26	2.11 ± 0.42	0.74 ± 0.17	I
3i-1000	1	D2-10	1.38 ± 0.30	1.25 ± 0.18	2.57 ± 1.55	1.35 ± 0.43	1.94 ± 0.89	1.08 ± 0.75	II
3i-1000	3	D2-10	**4.25 ± 0.75**	0.87 ± 0.07	**4.77 ± 0.95**	**1.86 ± 0.19**	1.05 ± 0.35	**3.07 ± 0.68**	II
3i-1000	5	D2-10	**13.62 ± 2.31**	**0.80 ± 0.08**	**7.70 ± 1.15**	**1.96 ± 0.25**	**0.56 ± 0.11**	**3.27 ± 0.73**	II
3i-1047	3	D2-10	1.85 ± 0.36	1.23 ± 0.25	-	-	-	-	II
3i-1047	10	D2-10	**3.79 ± 0.97**	0.74 ± 0.11	-	-	-	-	II
3i-1103	1	D2-10	2.40 ± 1.25	0.86 ± 0.10	4.62 ± 1.98	3.73 ± 2.35	2.17 ± 0.70	4.69 ± 3.10	II
3i-1103	3	D2-10	4.65 ± 2.33	**0.61 ± 0.11**	5.88 ± 1.92	4.15 ± 1.29	1.36 ± 0.30	8.85 ± 2.76	II
3i-1103	5	D2-10	**10.14 ± 2.15**	**0.46 ± 0.09**	**18.92 ± 4.73**	**3.31 ± 0.59**	1.57 ± 0.40	**11.56 ± 3.26**	II
CCK-8	0.175	D2-D12	0.97 ± 0.10	1.42 ± 0.22	1.20 ± 0.33	0.98 ± 0.19	1.23 ± 0.28	0.74 ± 0.16	III
CCK-8	0.35	D2-D12	0.73 ± 0.13	1.33 ± 0.13	0.74 ± 0.17	0.90 ± 0.19	1.09 ± 0.25	0.72 ± 0.17	III
CCK-8	0.175	D2-D5	0.82 ± 0.04	1.13 ± 0.07	1.09 ± 0.25	1.01 ± 0.23	1.33 ± 0.27	0.87 ± 0.15	III

Treatment	Dose (µM)	Treatment Window	%venGFP	venGFP MFI	%atrRFP	atrRFP MFI	%atrRFP/ %venGFP	atrRFP MFI/venGFP MFI	Study
CCK-8	0.35	D2-D5	1.09 ± 0.16	1.07 ± 0.12	1.26 ± 0.20	1.30 ± 0.26	1.29 ± 0.32	1.19 ± 0.14	III
CCK-8	0.175	D6-D9	0.91 ± 0.11	1.01 ± 0.10	1.08 ± 0.28	1.26 ± 0.39	1.31 ± 0.47	1.43 ± 0.62	III
CCK-8	0.35	D6-D9	0.77 ± 0.09	1.02 ± 0.05	0.79 ± 0.14	1.35 ± 0.42	1.03 ± 0.12	1.37 ± 0.48	III
CCK-8	0.175	D9-D12	0.86 ± 0.06	1.26 ± 0.11	1.69 ± 1.15	1.35 ± 0.40	1.88 ± 1.21	1.13 ± 0.38	III
CCK-8	0.35	D9-D12	0.91 ± 0.06	0.95 ± 0.10	1.35 ± 0.55	1.45 ± 0.24	1.50 ± 0.57	1.60 ± 0.36	III

5.3 STUDY III

In Study III, a novel chamber-specific marker of the embryonic and neonatal heart was characterized. proCck was first identified as a gene-of-interest based on analysis of mRNA-seq data of Tbx5 (-/-), Nkx2-5 (-/-), and Tbx5(-/-)/Nkx2-5(-/-) mESC-derived cardiomyocytes (Luna-Zurita et al., 2016). This analysis suggested that proCCK is a transcriptional target of TBX5, but not NKX2-5 in embryonic cardiomyocytes, in contrast to peptide hormones Nppa and Nppb. Interestingly, embryonic expression of proCck in the heart had not previously been characterized and was examined by qRT-PCR, whole mount *in situ* hybridization, and immunohistochemistry. proCck expression was further examined by conducting qRT-PCR analysis of primary pharyngeal arches (PA1), secondary pharyngeal arches (PA2), embryonic atria, and embryonic ventricles. Strikingly, proCck was upregulated in the developing cardiac chambers compared to PA1 and PA2, which contain undifferentiated progenitor cells. This occurred in concert with the onset of expression of the core cardiac transcription factors Tbx5, Gata4, and Nkx2-5. In order to show the anatomical localization of gene expression, whole mount *in situ* hybridization was performed in conjunction with optical projection tomography of embryonic and neonatal hearts. These experiments are summarized in Table 8. Interestingly, proCck mRNA was detected at the linear heart tube stage of mouse embryos, indicating its presence in the earliest stages of cardiogenesis. Furthermore, chamber-specific expression patterns were observed throughout embryogenesis, including expression in the regions of the forming interventricular septum, an area whose formation is dependent on the presence of TBX5. Neonatal proCck was mostly restricted to the atria and the left ventricle, including a region in the right atria encompassing the sinoatrial node. Furthermore Cckar and Cckbr mRNA localization were examined, indicating that CCKBR is the predominant receptor in neonatal hearts. To identify potential regulators of proCck expression, unbiased DNA motif analysis was performed for core cardiac transcription factors and transcriptional effectors of developmental signaling pathways. In addition to confirming the enrichment of TBX5 DNA binding motifs near the proCck transcriptional start site, *in silico* analysis implicated MEF2 factors as potential regulators of proCck transcriptional

promoter	cell/tissue type	stimulus	normalized luciferase
proCck 2500bp	HEK293	no serum + no TF	1
proCck 2500bp	HEK293	no serum + Tbx5	**1.86 ± 0.31**
proCck 2500bp	HEK293	no serum + Mef2c	**2.65 ± 0.26**
proCck 2500bp	HEK293	no serum + Gata4	1.16 ± 0.08
proCck 2500bp	HEK293	no serum + Tbx5 + Mef2c	**3.07 ± 0.63**
proCck 2500bp	HEK293	no serum + Tbx5 + Gata4	1.23 ± 0.08
proCck 2500bp	HEK293	no serum + Mef2c + Gata4	**1.96 ± 0.19**
proCck 2500bp	HEK293	no serum + Tbx5 + Mef2c + Gata4	**1.52 ± 0.08**
proCck 2500bp	HEK293	serum + no TF	1
proCck 2500bp	HEK293	serum + Tbx5	1.27 ± 0.07
proCck 2500bp	HEK293	serum + Mef2c	**2.53 ± 0.18**
proCck 2500bp	HEK293	serum + Gata4	1.27 ± 0.08
proCck 2500bp	HEK293	serum + Tbx5 + Mef2c	**1.67 ± 0.12**
proCck 2500bp	HEK293	serum + Tbx5 + Gata4	1.08 ± 0.14
proCck 2500bp	HEK293	serum + Mef2c + Gata4	**2.13 ± 0.12**
proCck 2500bp	HEK293	serum + Tbx5 + Mef2c + Gata4	1.39 ± 0.02
proCck 2500bp	neonatal ventricular cardiomyocytes	control	1
proCck 2500bp	neonatal ventricular cardiomyocytes	endothelin-1 100nM	**2.73 ± 0.16**
proCck 2500bp	neonatal ventricular cardiomyocytes	isoprenaline 100nM	2.01 ± 0.37

Table 10. Regulation of proCck transcription as detected in reporter constructs in HEK cells. Results from Study II demonstrate the regulation of proCck regulatory elements in cell-based models by exogenous stimuli and the core cardiac transcription factors Mef2c and Tbx5. procholecystokinin (proCck), Transcription factor (TF), T-box transcription factor 5 (Tbx5), Myocyte enhancer factor 2c (Mef2c), GATA transcription factor 4 (Gata4), Statistically significant changes are underlined in **bold**.

activity. In order to study regulatory regions of proCck in more detail, a 2500bp region flanking the proCck transcriptional start site (TSS) was cloned upstream of a luciferase reporter gene, and reporter gene assays were conducted in conjunction with overexpression of transcription factors Tbx5, Mef2c,

and Gata4 (Results shown in Table 10). These experiments confirmed that proCck is activated by TBX5 and MEF2C *in vitro*, but not by the Gata4 transcription factor. Intrigued by this finding, regulation of the 2500bp proCck-luciferase construct was measured in response to exogenous stimulation by ET-1 and isoprenaline in primary neonatal rat cardiomyocytes (Results shown in Figure 9). Interestingly, the proCck regulatory region was activated by both ET-1 and isoprenaline, suggesting its relevance in the disease context post-MI, a setting characterized by upregulation of both endothelin and adrenergic signaling. In order to confirm that this upregulation also occurs from the endogenous proCck locus, qRT-PCR was performed in primary neonatal cardiomyocytes following ET-1 treatment and cyclic mechanical stretch. This demonstrated that ET-1 stimulation increased endogenous proCck mRNA, suggesting that expression from the endogenous locus exhibits similar responses to pathological stimuli as the 2500bp regulatory region investigated in reporter assays. Emboldened by these findings, endogenous proCck mRNA was also examined in the left ventricular wall of rats subjected to myocardial infarction (Results shown in Figure 9). Surprisingly, proCck was downregulated in this context, suggesting complex regulation of endogenous proCck mRNA in response to MI. Importantly, no additional time points were measured post-MI, and it is therefore not known if this decline is persistent or transient. Due to its differential expression during development, we also sought to examine whether exogenous CCK-8 influenced the differentiation of pluripotent stem cells to cardiomyocytes, using the differentiation assay developed in Study I (Results shown in Table 8). These experiments demonstrated that exogenous CCK-8 did not have an effect on the differentiation of pluripotent stem cells to cardiomyocytes, leaving the precise physiological role of embryonic CCK as-yet-undiscovered.

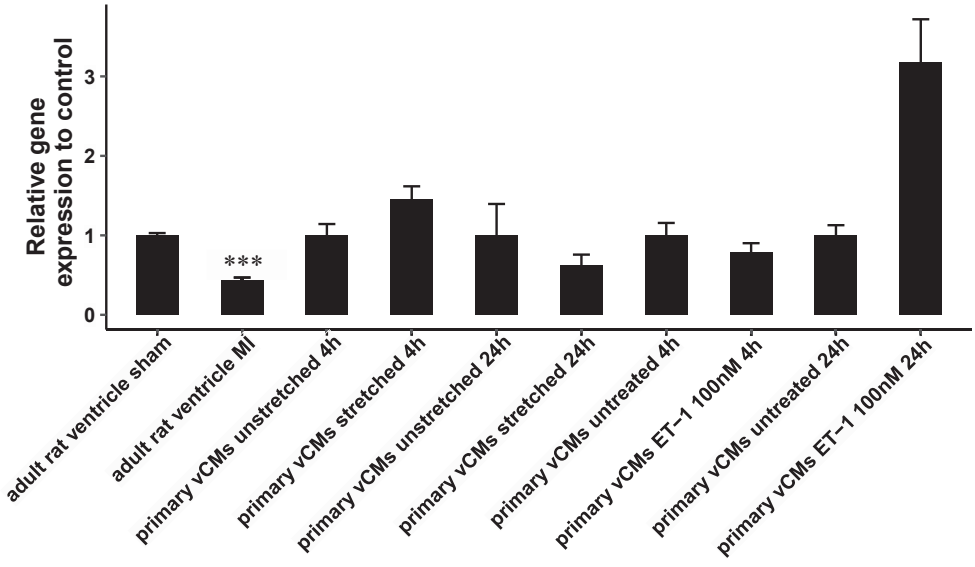

Figure 9. Regulation of proCck transcription by disease stimuli. Data is presented as mean + SEM (normalized to control). ***p<0.001 (T-test). mRNA levels of proCck were measured in ventricular tissue following acute myocardial infarction in adult rats. Additionally, proCck mRNA levels were measured in primary ventricular cardiomyocytes isolated from neonatal rats and subjected to cyclic mechanical stretch or cultured in the presence of endothelin-1 (100nM).

6 Discussion

6.1 Main findings and relation to previous studies

6.1.1 STUDY I

Study I describes the use of a genetic approach to monitor the differentiation of cardiomyocyte subtypes. Previous efforts have utilized similar strategies to detect the differentiation of atrial and ventricular cardiomyocytes, respectively. These have included the generation of separate pluripotent reporter lines for each subtype lineage (Josowitz et al., 2014; Lee et al., 2012; Moore et al., 2004; Müller et al., 2000), in addition to the development of dual reporter lines using distinct markers (Schwach et al., 2017; Yamauchi et al., 2018). However, the reporter line described in study I has several differences from the cell lines described in those studies. Firstly, study I utilized an alternative combination of markers than those used by others, including an exogenous quail transgene thought to be the only known atrial-specific regulatory element which maintains specificity to atrial myocardium during both embryonic and adult stages. The qRT-PCR analysis presented in study I confirmed the lack of a robust endogenous marker of atrial development in the murine model. Furthermore, previously described dual reporter lines relied on a negative selection strategy by combining a pan-cardiac reporter (Nkx2-5) with a subtype-specific reporter (Schwach et al., 2017; Yamauchi et al., 2018). Furthermore, published reporter lines lacked *in vivo* validation, and study I is the first study to demonstrate the validation of reporter lines by conducting laser-assisted morula injection to produce chimeric mouse embryos. Thus, study I is the first report of a dual reporter cell line for atrial and ventricular lineages with extensive *in vivo* marker validation.

Study I further describes the development of stage-specific screening assays for the assessment of differentiation modulators and identification of teratogenic compounds. *In vivo* teratogen screening represents a significant financial burden for pharmaceutical and chemical industries, and this cost is carried to public healthcare systems in the form of increased drug prices (Beekhuijzen, 2017). *In vitro* teratogen screening platforms have the potential to partially reduce this burden, and efforts to generate representative models have been described previously (Scholz et al., 1999; Seiler & Spielmann, 2011). Importantly, study I presents the first *in vitro* screening platform for the identification of teratogens which affect anterior-posterior patterning of multipotent progenitors by using a novel dual reporter line for atrial and ventricular cell fates (venGFP/atrRFP). As anterior-posterior patterning is a critical step during mammalian development, this assay might allow for the detection of teratogens not uncovered by previous assays. To this end, Study I described the effects of retinoids, known mediators of anterior-posterior patterning of the heart, on reporter cell patterning and differentiation (Keegan et al., 2005; Ryckebusch et al., 2008; Xavier-Neto et al., 1999). Interestingly, study I described more pronounced teratogenic effects for dual RAR/RXR agonists than for single RXR agonists, alluding to non-redundant effects of RAR and RXR during anterior-posterior patterning and serving as proof-of-concept for use of reporters of anterior-posterior patterning to uncover teratogens acting via distinct mechanisms. The use of reporter genes (as opposed to counting of beating foci) in order to determine teratogenicity also holds promise for increasing the throughput of in vitro reproductive toxicity screening.

Previously described teratogenic screening assays have incorporated long treatment windows encompassing varied developmental stages to identify differentiation modulators (Scholz et al., 1999; Seiler & Spielmann, 2011). In contrast, the assays developed in study I utilized shorter, more defined compound addition windows than those used in previous assays, and cell populations present at

compound addition points were characterized by qRT-PCR. In addition to allowing for the identification of novel teratogens, these defined treatment windows could facilitate the identification of novel mechanisms-of-action of teratogenic compounds. Furthermore, due to the relevance of differentiation mechanisms to the development of targeted regenerative therapies, this screening system could also be utilized to identify cardiogenic small molecules for the treatment of myocardial infarction, as described in study II.

6.1.2 STUDY II

Screening campaigns to identify compounds which promote the differentiation of cardiomyocytes from stem cells have been previously conducted by others and led to the identification of cardiogenic compounds (Table 6). Among these hit compounds exist chemical modulators of developmental signaling pathways which might have limited use in the clinical setting due to non-specific effects outside the cardiovascular system. Study II sought to identify cardiogenic activity among a family of compounds previously shown to interfere with the interaction of core cardiac TFs GATA4 and NKX2-5. By utilizing the dual reporter line generated in study I, this led to the identification of several compounds which preferentially activated atrial and ventricular reporters, respectively. To date, there have been no other reports of screening campaigns to identify specific chemical activators of atrial or ventricular reporters.

The direct targeting of TFs (excluding nuclear receptors) to induce cardiomyocyte differentiation has not been described, though they are largely considered to be non-druggable targets. In study II, immunoblotting suggests that compound treatment of differentiating pluripotent stem cells leads to upregulation of a previously unidentified 70kDa GATA4 molecular form, and downregulation of the 50 kDa GATA4 molecular form. Furthermore, the identification of an acetyl-lysine like fragment in active compounds, Bio-ID analysis of GATA4 interactions with bromodomain-containing proteins, and reporter gene assays with BET bromodomain inhibitors indicate suggestive mechanisms-of-action of 3i-1000 in its mediation of transcriptional activity by modulating bromodomain-containing proteins. These provoke interesting follow-up studies involving the further investigation of cardiogenic compounds on the modulation of epigenetic proteins to control chamber-specific gene expression.

Cardiogenic compounds without deleterious effects on non-cardiac tissues might hold promise for the treatment of heart failure. Importantly, cardiogenic compounds might induce endogenous cardiac progenitors, which normally differentiate into stromal cells, to differentiate into beating heart muscle. Alternatively, these compounds could be used to interrupt the increased activity of core cardiac transcription factors following myocardial infarction. Indeed, one compound tested in study II has shown beneficial effects in preclinical animal models of myocardial infarction, suggesting the suitability of *in vitro* phenotypic screening to identify compounds with *in vivo* bioactivity (Kinnunen et al., 2018).

6.1.3 STUDY III

CCK has long been known as a peptide hormone present in the gut and central nervous system (Rehfeld, 1978). Though its expression has recently been observed in cardiac tissues (Goetze et al., 2015), upstream regulators of its tissue-specific expression have not previously been identified. The significance of transcriptional regulation of peptide hormones secreted by the heart is exemplified by the cardiac natriuretic peptides Nppa and Nppb. Transcriptional regulation of these peptides is governed by the core cardiac transcriptional network, and their secretion by the heart is an important

marker during heart failure (Durocher et al., 1996; Durocher et al., 1997; Magga et al., 1997; Majalahti et al., 2007; Pikkarainen et al., 2003). The description of cardiac transcriptional regulation by the gut hormone proCCK in Study III mirrors the extensive work that has been performed on Nppa and Nppb, but with important differences. Similar to Nppa and Nppb, study III reports the expression of proCck mRNA in the newly formed atrial and ventricular chambers of embryonic hearts, and the absence of expression in undifferentiated cardiac progenitors. Therefore, proCck could be used as a marker of newly differentiated cell populations in different contexts, such as native embryogenesis, stem cell differentiation, and cellular reprogramming. Interestingly, proCck mRNA was upregulated in embryonic ventricular tissue compared to atrial tissue, similar to both Nppa and Nppb at this stage. In this line, proCck was detected by whole mount *in situ* hybridization as early as the linear heart tube stage, and later showed restricted expression in anatomical subdomains of the heart. Interestingly, neonatal expression of proCck and its receptors was mostly confined to the atria, consistent with previous reports that exogenous CCK regulates heart rate, both *in vivo* and *ex vivo* in cardiac explants (Janssen et al., 1991; Marker & Roberts, 1988).

Whereas embryonic expression of proCck appeared to mirror that of Nppa and Nppb, it was shown in study III to be under the transcriptional control of distinct members of the core cardiac transcription network and to respond differently to disease stimuli. In contrast to Nppa and Nppb, proCck was shown to be transcriptionally activated by TBX5 and MEF2C, but not by GATA4. Furthermore, whereas Nppa and Nppb were upregulated in the left ventricular wall post-myocardial infarction, proCck was markedly downregulated in this tissue. However, transcription of all three genes were upregulated by exogenous delivery of Endothelin to cultured primary cardiomyocytes. These results indicate divergent transcriptional regulation of proCck, a peptide hormone, from the canonical cardiac natriuretic peptides Nppa and Nppb. As serum CCK has recently been proposed as a marker of mortality in heart failure patients (Goetze et al., 2016), this divergent regulation could have relevance to heart failure progression and nefarious transcriptional remodeling. Interestingly, the role of TBX5 in heart failure progression has not been described. Finally, no effects of exogenous delivery of CCK-8 on the differentiation of cardiac progenitors to atrial and ventricular cardiomyocyte subtypes were observed, and the function of this peptide during embryogenesis remains unknown.

6.3 Study limitations and future prospects

6.3.1 STUDY I

Study I details a novel reporter line that can be used as a tool to assess the differentiation of atrial and ventricular cardiomyocytes, and how it could be used as a method to detect teratogens. Importantly, Study I does not represent a mature assay ready for wider deployment. In order for this assay to become established as a mainstream tool during preclinical drug development, it would require further validation by screening of a larger number of known and unknown teratogens in more time windows in order to estimate the applicability domain of the assay. These results would have to be correlated with *in vivo* testing in experimental animal models. Prior to undertaking these ambitious follow-up studies, it might be worthwhile to further optimize the *in vitro* screening system to increase both sensitivity and throughput, perhaps utilizing high-content imaging as an endpoint and incorporating robotic compound addition.

Importantly, study I utilized mouse, rather than human PSCs for the generation of reporter cell lines. A key advantage of this approach is that it allowed for the generation of chimeric reporter mice to assess the correct expression patterns of reporter genes in developing cardiac chambers. However, human PSC-based assays might be more relevant to the assessment of teratogenic activity in humans.

Indeed, thalidomide has teratogenic activity in humans but not in mice, explaining the failure to identify its teratogenic properties prior to use in humans (Kazuki et al., 2016). However, this is due to differential metabolism of compounds by human and mouse hepatic tissue, and even human PSC cardiac differentiation models would be unable to detect these differences (Kazuki et al., 2016). Indeed, early developmental steps are widely conserved in vertebrate species, so this may not be a pertinent issue.

6.3.2 STUDY II

Study II detailed the effects of a family of GATA4-targeted compounds on cardiac differentiation of PSCs. Though the present study detailed activation of cardiac reporter genes, it is unknown to what degree the cells generated represent fully mature cardiomyocytes. Indeed, cardiomyocytes produced from PSCs are known to represent immature, embryonic-like cells unless transplanted into the adult mouse heart (Cho et al., 2017). Follow-up studies consisting of detailed electrophysiological characterization of chemically-induced PSC-derived cardiomyocytes could allow for assessment of their functional maturity. Furthermore, though several compounds were identified in this study with activity *in vitro*, *in vivo* testing of these compounds was not performed. It would be interesting to test a subset of active compounds in mouse models of MI and compare levels of *in vitro* potency to *in vivo* potency. Additionally, it would be interesting to determine effects of compounds in conjunction with exogenous cell injection of PSC-derived CPs or CMs to the infarcted heart.

In study II, only compound derivatives of GATA4-targeted compounds were tested. Due to the wide number of molecular entities involved in cardiogenesis, there are likely many druggable targets capable of modulating cell fate decisions of CPs. It would thus be worthwhile to perform compound screening of more diverse chemical compound libraries to uncover novel chemical entities which regulate atrial and ventricular differentiation. Increasing the throughput of the screening assay would potentially allow for screening of many thousands of compounds. In addition to chemical compounds, siRNA or CRISPR-based screening could also be performed in order to identify novel molecular targets involved in atrial or ventricular specification.

Our BioID experiments implicated interaction of GATA4 with bromodomain-containing proteins such as BRD4. Due to the need for large amounts of cell material, these experiments were performed in the non-cardiac HEK cell line. Importantly, this cell line might provide a limited representation of the GATA4 interactome. It would be interesting to perform the same experiments in CP or CMs in order to determine the cardiac-specific interactome of GATA4. However, this would most likely require the generation of transgenic PSC or rodent lines in order to achieve ubiquitous overexpression of BioID components and obtain sufficient cell material for mass spectrometry.

6.3.2.1 Chemical modulation of bromodomain-containing proteins and the epigenome: a way to chemically target transcription factors?

Though TFs are integral to development, they are not easily targetable by chemical compounds. In study II, we identified GATA4/BRD4 crosstalk and its modulation by BET bromodomain inhibitor JQ1 and GATA-targeted compound 3i-1000. Recently, bromodomain-targeted compounds have been developed which allow the modulation of master TF activity at the transcriptional level. In a landmark study, a small molecule was identified which binds to the bromodomain containing 4 (BRD4) bromodomain and competes for access to acetylated histones, illustrating the possibility of inhibiting protein-protein interactions of epigenetic reader proteins within the native chromatin context (Filippakopoulos et al., 2010). Based on this approach, several small molecule inhibitors of

bromodomain-histone interactions have been developed, and biological effects of bromodomain inhibitors have been extensively reported during the past decade, though the majority of these studies are in cancer models. JQ1, a bromodomain and extraterminal domain (BET) bromodomain inhibitor, led to decreased transcription of the MYC proto-oncogene, basic helix-loop-helix transcription factor (MYC) in multiple myeloma cells, and a further decrease in transcription of MYC-target genes (Delmore et al., 2011). Effects of JQ1 were later attributed to loss of BRD4 at super-enhancers, causing preferential downregulation of genes with super enhancers, such as MYC (Loven et al., 2013). Thus, it is possible to inhibit TFs indirectly at the transcriptional level by modulating bromodomain-enhancer interactions. This mechanism of JQ1 mediated disruption of transcription at the c-Myc super enhancer was also apparent in merkel cell carcinoma, displaying the potential universality of this approach (Sengupta et al., 2015). However, resistance to JQ1 in leukemic stem cells was shown to be due to a subsequent compensatory increase in Wnt signaling (Fong et al., 2015; Rathert et al., 2015). The BET inhibitor RVX2135 was shown to induce similar transcriptional effects as that of histone deacetylase inhibitors, suggesting similar outcomes may be achieved by inhibiting bromodomain:acetylated histone interactions as inhibiting the removal of acetylated histones (Bhadury et al., 2014). This was partially attributed to perturbation of the positive transcription elongation factor (p-TEFb) transcriptional pause mechanism by BET inhibition, rather than to decreased recruitment of RNA polymerase II, and they speculate that BET inhibition will have different effects in different cell types due to the altered placement of histone marks (Bhadury et al., 2014). In a mouse model of pancreatic ductal adenocarcinoma, combinatorial actions of JQ1-mediated bromodomain inhibition and HDAC inhibition were observed (Mazur et al., 2015). In a somewhat contrarian study, it was shown that BRD4 inhibitors led to an increase in BRD4 protein accumulation causing them to have modest effects on Burkitt's lymphoma (Lu et al., 2015). The authors thereafter developed proteolysis-targeting chimera compounds which bind to the bromodomain-containing site of BRD4 and lead to ubiquitination and degradation (Lu et al., 2015). This approach could be further exploited to increase effects of bromodomain inhibitors. Interestingly, inhibition of the bromodomain of CBP/EP300 was shown to promote the reprogramming of fibroblasts to iPSCs (Ebrahimi et al., 2019).

Recently, inhibition of BET bromodomains has been proven to ameliorate heart failure in small animal models. In a pioneering study, BET bromodomain inhibitor JQ1 prevented the induction of pathological gene expression programs and pathological remodeling in both *in vitro* and *in vivo* models of heart failure (Anand et al., 2013; Duan et al., 2017). BET bromodomain inhibitor JQ1 inhibited angiotensin II-induced activation of super-enhancers of atherosclerosis and growth-factor signaling genes, reducing hypertension, hypertrophy, and inflammation (Das et al., 2017). There are so far no reports of bromodomain inhibition during cardiomyocyte differentiation. However, the BET bromodomain inhibitor JQ1 inhibited the self-renewal of mesenchymal stem cells via the downregulation of Wnt signaling (Alghamdi et al., 2016). Additionally, JQ1 enhanced the differentiation of mouse pluripotent stem cells to the endodermal lineage (Gonzales-Cope et al., 2016). In the same study, the authors observed a steady decrease in both histone acetylation and Brd4 during normal differentiation, suggesting that perturbation of these protein dynamics can cause an increase in cell differentiation (Gonzales-Cope et al., 2016). Thus, bromodomain inhibitors, epigenetic inhibitors, and other modulators of transcription could represent therapeutic avenues for the specific regulation of cardiovascular functional outcomes by context-dependent mechanisms. Our identification of GATA4/Bromodomain interactions and their chemical modulation by JQ1 and 3i-1000 could be a gateway to the exploration of these mechanisms to ameliorate cardiovascular disease.

6.3.3 STUDY III

Study III consisted of the characterization of proCCK expression in cardiac tissues and the identification of TBX5 and MEF2C as the major transcriptional regulators responsible for its expression in the heart. The identification of TBX5 and MEF2C as transcriptional regulators of proCck was based on analysis of unbiased mRNA-sequencing and DNA motif analysis. An important consideration is that DNA motif analysis is not in itself proof of biological relevance or transcriptional activity. However, motif analysis was used to identify potential transcriptional regulators (MEF2C, TBX5), and conclusions regarding their activity were based on reporter gene assays involving MEF2C and TBX5 overexpression. Though *in silico* analysis suggested that motifs for other TFs were also present (albeit at lower levels) in the proCck regulatory region, these were not further explored. It is highly likely that some of these are also transcriptional regulators of proCck, and it would be interesting to test these in follow-up studies.

In study III, only a single proCck regulatory region was examined to ascertain transcriptional regulation in reporter gene assays. Though the 2500bp regulatory region encompassing the proCck transcriptional start site described in study III was responsive to TBX5, MEF2, ET-1, and Isoprenaline, there might be distal regulatory regions which also regulate proCck transcription. Importantly, distinct regulatory regions governing fetal and adult transcription of Nppa have been described (Horsthuis et al., 2008). Further examination of proCck regulatory regions could provide more information about its transcriptional regulation, and candidate regulatory regions could be identified by examination of publicly available ChIP-seq data.

In contrast to other cardiac peptides (Nppa/Nppb), we observed downregulation of proCck mRNA levels following myocardial infarction. However, only a single timepoint was examined in this analysis, a limitation of the study. Additionally, our ISH analysis identified that proCck expression is more predominant in atria than ventricles, and no atrial samples were analyzed from infarcted animals. In order to draw more robust conclusions about proCck mRNA changes in the infarcted heart, it would be better to analyze samples from all four chambers of the heart at different timepoints following MI. This would allow appreciation of the spatiotemporal regulation of proCck mRNA in the MI model. Additionally, it would be interesting to examine TBX5/MEF2C binding in these samples.

Though Study III provided insight into expression domains and transcriptional regulation of CCK, it failed to identify a physiological role of secreted embryonic CCK peptide. Treatment of differentiating PSCs with exogenous peptide did not appear to alter differentiation dynamics, as measured by expression of atrial and ventricular reporters. Though this is evidence that CCK is not a differentiation modulator, it is not altogether conclusive. Importantly, it has previously been shown that cardiac processing of the CCK peptide is distinct from processing in other tissues. However, the shorter CCK-8 peptide (important in digestive and nervous systems) is currently the only commercially available version, and this was used in differentiation assays in the present study. Should the longer, cardiac version become commercially available, it would be interesting to examine its capacity to modulate cardiac differentiation. Alternatively, a transgenic cassette could be overexpressed in mESCs to see effects on differentiation when processed endogenously. Indeed, though transcriptional regulation was characterized in detail, no work was performed on post-translational processing of proCCK, and it is unknown if the cardiac form of CCK in the adult heart is also the predominant form in embryonic hearts.

7 Summary and conclusions

Collectively, this dissertation describes the experimental investigation of cell-subtype specific gene regulation during cardiovascular lineage diversification and cell differentiation. To explore this topic, a novel cell-based model was generated using genome editing, and this reporter system was later utilized to explore modulation of atrial and ventricular cardiomyocyte differentiation. Additionally, proCck was characterized as a novel chamber-specific marker in the embryonic and postnatal heart under the regulation of TBX5/MEF2 transcription factors.

The principle findings of the work may be summarized as:

1. Genome editing of pluripotent stem cells is a viable strategy for the *in vitro* study of anterior-posterior patterning of multipotent cardiac progenitors. Specifically, generation of a knock-in allele at the Myl2 locus led to specific activation of a fluorescent reporter in stem cell- and embryo-derived ventricular cardiomyocytes both *in vitro* and *in vivo*. qRT-PCR analyses confirmed previous reports of the lack of endogenous atrial-specific genes, but this was circumvented by the utilization of a novel SMyHC3-TdTomato transgene for dual-reporter cell generation. Detection of reporter alleles by automated systems increased the suitability of dual reporter pluripotent stem cells for chemical screening.

2. Dual reporter pluripotent stem cells could be utilized to conduct differentiation assays for the identification of compounds that affect anterior-posterior patterning. Consistent with expectations, retinoids exhibited stage-specific effects on the expression of atrial and ventricular gene expression. High concentrations of retinoic acid (10μM) added prior to the onset of spontaneous beating resulted in a near total loss of both atrial and ventricular reporter gene expression, consistent with reported effects in zebrafish embryos. However, decreased concentrations of retinoic acid (1μM) led to increases in the proportion of atrial cells relative to ventricular cells, similar to the expansion of the atria in embryological studies in published reports, suggesting that the *in vitro* dual reporter model can reproduce phenotypes observed *in vivo*. Chemical modulators of Wnt and TGFβ signaling pathways were tested in a high-throughput variation of the assay and were shown to increase ventricular differentiation of PSCs. Thus, chemical screening within reporter PSCs is a valid avenue for the identification of novel compounds which affect atrial and ventricular gene expression, important for both reproductive toxicity testing and the identification of regenerative small molecules.

3. In addition to chemical modulators of upstream developmental signaling pathways, compounds targeted to downstream TFs also modulated atrial and ventricular reporter gene expression. Derivatives of 3i-1000, a compound which inhibits the interaction of core cardiac TFs GATA4 and NKX2-5, were tested for effects on atrial and ventricular reporter expression in differentiating PSCs. A subset of compounds led to differentional activation or repression of atrial and ventricular reporters and served as the basis for identification of a structure-activity relationship. A component of this process was the increase in a previously uncharacterized molecular form of GATA4 during chemically-induced differentiation. Compound structure led to the investigation of epigenetic mechanisms, and chemical inhibitors of BET bromodomains were shown to modulate GATA4-dependent regulation of a chamber-specific reporter construct.

4. Analysis of published mRNA-seq datasets indicated the gut/neurohormone cholecystokinin as a direct transcriptional target of TBX5 in pluripotent stem cell-derived cardiomyocytes. Whole mount in situ hybridizations in embryos revealed CCK expression at the linear heart tube stage and the early four-chambered heart. Expression of proCck in the neonatal heart was mostly restricted to the atria

and left ventricle, whereas expression of the CCKB receptor was mostly restricted to the atria (CCKA receptor mostly absent). DNA motif analysis revealed the presence of TBX5 motifs near the proCck transcriptional start site, in addition to those of the core cardiac TF MEF2C. Reporter gene assays revealed that both MEF2C and TBX5 activate the proCck regulatory region, and this promoter sequence was also responsive to both ET-1 and adrenergic stimulation. Following MI, proCck mRNA levels were downregulated in the left ventricular wall, in contrast to natriuretic peptides Nppa and Nppb. Exogenous delivery of CCK-8 peptide to differentiating PSCs did not affect the differentiation of atrial and ventricular cardiomyocytes in the dual reporter differentiation assay

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